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Hyperactivated motility in human sperm regulation, function and significance in IVF

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Contents

Declaration

The data used in Chapter 7 of this thesis was gathered jointly by myself and Wardah Al-Asmari, whom I would like to thank for the fun we had together, and wish the best of luck for her PhD. All remaining data was gathered by me alone, and I am the sole author of the text. I have personally consulted all references and I have not submitted this thesis previously for any other degree.

Erica Foster

Acknowledgements

I would like to offer my most sincere gratitude to all the people who have helped me over the past two years. This includes everyone in the MACHS labs (plus Steve Publicover in Birmingham) and all the staff of Ward 35 Assisted Conception (in particular, of course, the embryology team).

I would especially like to thank Christopher Barratt, Wardah Al-Asmari, Steven Mansell, Mum, Dad, Syl and Michael Henderson.

Summary

Hyperactivation is a movement pattern elicited by sperm in the female reproductive tract. The transition to hyperactivated movement is characterised by an increase in amplitude and a decrease in symmetry of flagellar bending. The resulting forceful, unequal waveforms produce a swimming style that is erratic and often non-progressive. Linked temporally with the process of capacitation, hyperactivation is thought be a pre-requisite for fertilisation. Its putative roles are in navigation and movement through the oviduct, infiltration of the cumulus oophorus and penetration of the zona pellucida. The majority of evidence for the requirement for mammalian hyperactivation has emerged from studies in the mouse and the hamster, however a number of clinical studies in humans have also suggested that its expression is linked to male fertility both in vivo and in vitro. The expression of hyperactivation appears to be controlled both by Ca^{2+} entry through plasma membrane CatSper channels and by Ca^{2+} release from intracellular stores. Using CASA, the efficacy of pharmacological agents known to influence hyperactivation was assessed, and a robust hyperactivation assasy was developed. The assay was used both to explore the incidence of hyperactivation in fertile and infertile populations of men, and to attempt to define the prognostic potential of a pre-IVF hyperactivation screen. Whilst sperm from men undergoing ICSI treatment exhibited lower levels of hyperactivation than sperm from men in whom no semen abnormalities could be identified, there was no correlation between hyperactivation and fertilisation rate in conventional IVF. In the final set of experiments, an attempt to affirm the role of store-operated Ca^{2+} entry in hyperactivation was made, however drugs known to modulate this pathway did not influence any sperm motility parameters.

List of abbreviations

2-APB: 2-aminoethoxydiphenyl borate

4-AP: 4-aminopyridine

AC: Adenylate cyclase

ALH: Amplitude of lateral head displacement

ANOVA: Analysis of variance

ATP: Adenosine-5'-triphoshphate

BCF: Beat cross frequency

BMI: Body mass index

BSA: Bovine serum albumin

cAMP: Cyclic adenosine monophosphate

CASA: Computer-assisted sperm analysis/analyser

CCD: Charge-couple device

CCE: Capacitative calcium entry

CICR: Calcium-induced calcium release

CM: Capacitating medium

CRAC: Ca^{2+}-release-activated current

DNA: Deoxyribonucleic acid

EGTA: Ethylene glycol tertaacetic acid

FISH: Fluoresence *in situ* hybridisation

FSH: Follicle stimulating hormone

GnRH: Gonadotrophin-releasing hormone

hCG: human chorionic gonadotrophin

HSA: Human serum albumin

HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

IBMX: 3-isobutyl-1-methylxanthine

ICSI: Intra-cytoplasmic sperm injection

IMSI: Intra-cytoplasmic morphology selected sperm injection

IVF: In vitro fertilisation

LIN: Linearity

MII: Meoisis II

NCB: Non-capacitating buffer

PKA: Protein kinase A

ROS: Reactive oxygen species

SCSA: Sperm chromatin structure assay

SERCA: sarco/endoplasmic reticulum Ca^{2+}-ATPase

SKF-96365: 1-[2-(4-Methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl-1*H*-imidazole hydrochloride

SOCE: Store-operated calcium entry

SPCA: Secretory pathway Ca^{2+}-ATPase

STR: Straightness

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

VAP: Average path velocity

VCL: Curvilinear path velocity

VSL: Straight line velocity

WHO: World Health Organisation

Chapter 1: Regulation and function of sperm motility and hyperactivation in humans

1.1 A sperm cell's journey to the oocyte

Crucial to achieving fertilisation in vivo is the ability of sperm to travel from their point of deposition in the female tract to the oviductal ampulla, where, if ovulation occurs, they will encounter the oocyte. Delivery of their genetic material involves an arduous journey for these cells, and although hundreds of millions of sperm may be ejaculated by the human male, only tens, or at the very most hundreds, will complete it (De Jonge, 2005).

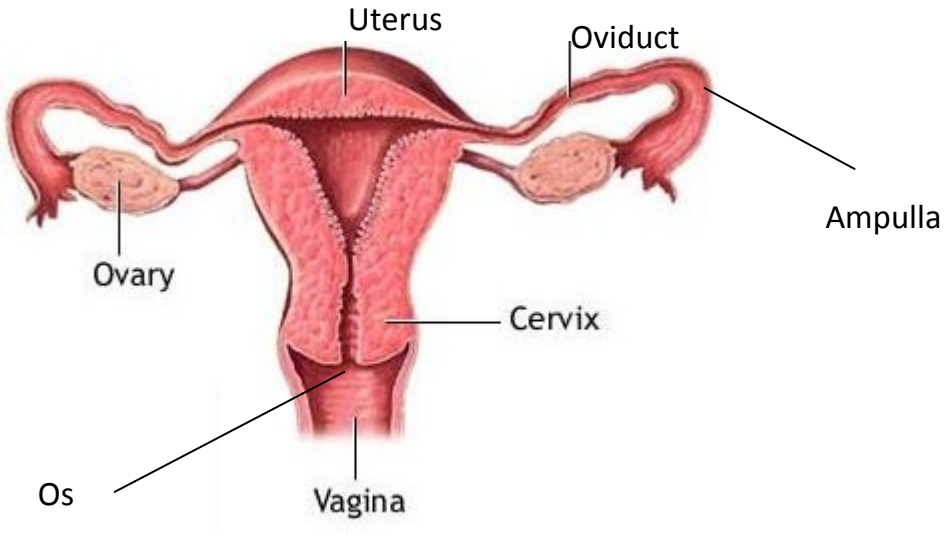

Figure 1.1: The female reproductive tract. Adapted from image on (www.nursingcrib.com, 2011).

Following spermatogenesis in the seminiferous tubules, human sperm are transported to and stored in the epididymis, the site at which they undergo their final stages of maturation (Bjorndahl *et al.*, 2010). At ejaculation during coitus they are expelled from the urethra in prostatic fluid. Together the sperm and prostatic components form the first portion of the ejaculate (Kvist, 1991). Large numbers of cells may be lost to backflow, but the remainder escape this hostile, acidic environment by quickly beginning to swim out from seminal plasma into the cervix via the external os, from which mucus to 'catch' the

sperm emanates (Suarez and Pacey, 2006). During the peri-ovulatory period, the cervical glands produce mucus which is more hydrated than at other times of the menstrual cycle (Morales *et al.,* 1993; Katz *et al.,* 1997). Despite this enhanced receptivity to sperm, only cells which are morphologically normal and moving with sufficient velocity in a forward progressive manner will adeptly traverse the cervix (Hanson and Overstreet, 1981; Katz *et al.*, 1990).

Contractile activity of the myometrium and/or the action of uterine cilia, may encourage the flow of sperm into the uterine cavity (Suarez and Pacey, 2006; Elder and Dale, 2011). To avoid attack by leukocytes, which will enter the uterus following ejaculation, it may be important for sperm to progress to the uterotubal junction quickly (Suarez and Pacey, 2006).

The lumen of the uterotubal junction is narrow, folded and contains oestrogen-dependent viscous mucous which flows in the opposite direction to sperm movement (Jansen, 1980; Bjorndahl *et al.*, 2010). Whilst sperm inherently swim against currents, the junction may represent an additional system by which to 'filter out' poorer quality sperm (Mortimer, 1997). Human sperm have been seen to bind intermittently to the epithelial cells in the isthmic portion of oviduct (Pacey *et al.,* 1995; Pacey *et al.,* 1995; Baillie *et al.,* 1997), an interaction which may both prolong sperm survival (Kervancioglu *et al.,* 1994) and limit their passage through the tubes, thereby reducing the incidence of polyspermy (Suarez and Pacey, 2006).

Components of follicular fluid or secretions from the mature ooctye and its vestments may then guide the sperm chemotactically towards the exact site of fertilisation (Eisenbach,

1999; Sun *et al.*, 2005). The time from insemination to fertilisation has been proposed to be at least one hour, and up to as many as five days (Suarez and Pacey, 2006).

1.2 Fertilisation

The in vivo fusion of the mature male and female gametes involves a sophisticated and highly ordered series of events. With respect to the role of the spermatozoon, completion of the following steps is crucial:

1. Acquisition of a 'capacitated' status, at some point between the cervix and the oviductal ampulla
2. Passage through the jelly-like matrix of the cumulus oophorus surrounding the oocyte
3. Specific binding to the zona pellucida, a glycoproteinous capsule which directly coats the oocyte
4. Exocytosis of acrosomal contents, a process which renders the sperm plasma membrane capable of interacting with the oolemma
5. Penetration of the zona pellucida
6. Binding to and fusion with the oolema

Compiled using information from (Johnson, 2007; Publicover *et al.*, 2007).

Following membrane fusion, the soluble sperm-oocyte activating factor, phospholipase zeta, promotes the opening of Ca^{2+} stores in the ooplasm which create calcium ion transients. The cortical granule reaction and vitelline block prevent polyspermy, the sperm

nucleus moves to the oocyte cortex and begins to decondense, and meiosis is reinitiated (Johnson, 2007).

The use of in vitro fertilisation entirely eliminates the barrier posed to sperm by the female reproductive tract. During conventional IVF, penetration of the cumulus oophorus and the zona pellucida by sperm are still absolute requirements for fertilisation. By employing intra-cytoplasmic sperm injection, these lattermost obstacles are also removed, allowing even the most severely compromised sperm access to the ooplasm.

1.3 Flagellar architecture and the controls of sperm motility

The flagellum, responsible for the propulsion of sperm through the female reproductive tract, comprises the connecting piece, midpiece, principal piece and end piece. Motility is generated by the axoneme, a structure which runs through the centre of the sperm tail and is supported by a protective cytoskeletal sheath (Figure 1.2).

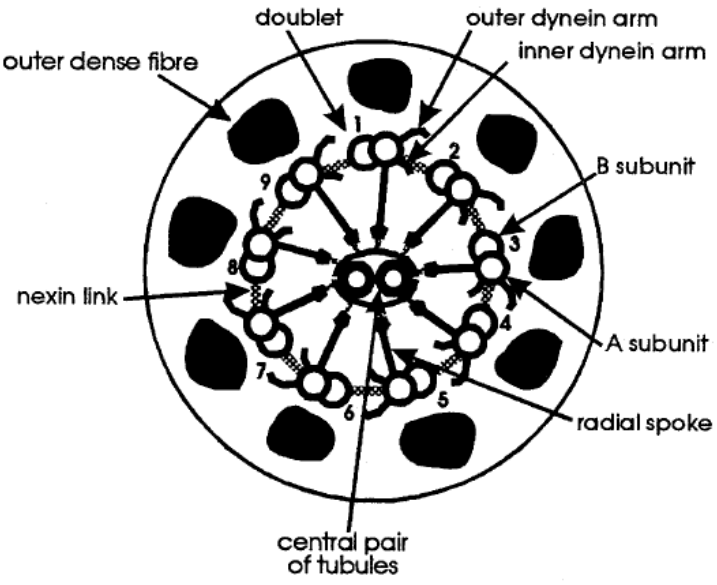

Figure 1.2: Cross section through the axoneme. From (Mortimer, 2000).

In a classical arrangement, two central single microtubules are surrounded by nine peripheral microtubule doublets (Inaba, 2003). Nexin links connect the doublets to each other, and radial spokes bridge the outer nine microtubules and the central pair (Mortimer, 1997). The connecting piece at the base of the flagellum is fused with outer dense fibres, linking the tail and the head (Fawcett, 1975). Movement is enabled by the accessory protein dynein, which forms distinct 'arms' on the peripheral doublets and whose ATPase activity provides the mechanical energy required to 'walk' one microtubule doublet along an adjacent one via the sequential making and breaking of attachments (Mortimer, 1997). The inner dynein arms confer flagellar waveform and beat symmetry, and the outer arms establish the maximal velocity of outer doublet movement (De Jonge and Barratt, 2006). Localised sliding of microtubules creates bending in the flagellum, and the asymmetry of the axoneme and the sequence of attachments result in waves that appear helical, but are not quite (Mortimer, 1997). Beat frequency is controlled at the base of the flagellum, and waves that propagate backwards to create forward propulsion (Mortimer, 1997; Oehninger and Kruger, 2007).

Sperm cells acquire the ability to swim during their transit through the epididymis, and motility becomes 'activated' when they come into contact with seminal plasma at ejaculation (Oehninger and Kruger, 2007). Extrinsic factors such as the extracellular ionic environment and specific components present in semen and the female reproductive tract are responsible for initiating and maintaining sperm motion.

Secreted by the prostate gland, and important for pH buffering in the vagina, the bicarbonate ion plays a crucial role in switching on and maintaining sperm motility (Wennemuth *et al.,* 2003), whose primary roles are outlined in the Figure 1.3.

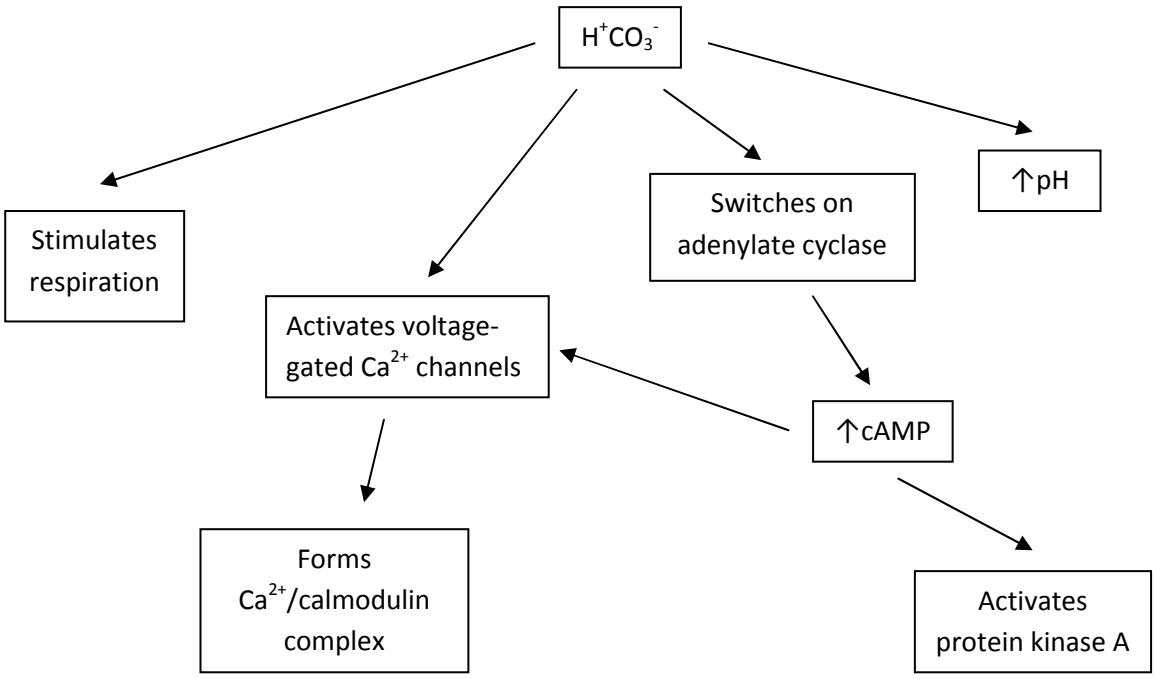

Figure 1.3: Biochemical control of sperm motility in humans. Compiled using information from (Luconi and Baldi, 2003; De Jonge and Barratt, 2006).

Cyclic AMP and Ca^{2+} are the key second messenger molecules involved in the initiation and regulation of sperm motility (Luconi and Baldi, 2003; Esposito *et al.*, 2004). The rise in pH promotes the opening of alkaline-dependent Ca^{2+} channels, which, along with Ca^{2+} flux through voltage-gated channels, raise $[Ca^{2+}]_i$. Calcium ions form a calcium/calmodulin complex which interacts with a number of enzymes, including cyclases, phoshatases and kinases (Jaiswal and Conti, 2003; Ignotz and Suarez, 2005; Marin-Briggiler *et al.*, 2005). cAMP activates protein kinase A (PKA), which phosphorylates serine or threonine reisdues on proteins. Eventually, this in turn results in the tyrosine phoshorylation of axonemal proteins, and axonemal sliding is translated into flagellar waves (Mortimer, 1997; Suarez, 2008). Protein kinase C may also be involved in the regulation of sperm motility (De Jonge and Barratt, 2006). The ATP requirement for sperm motility can be derived from both

oxidative phosphorylation and anaerobic glycolysis, with the former maximising output per glucose molecule, but the latter minimising the production of membrane-damaging reactive oxygen species (Nascimento *et al.*, 2008; Storey, 2008).

1.4 Hyperactivation

Microscopic observation of a normal fresh semen sample reveals activated cells travelling in a forward progressive, linear trajectory (Robertson *et al.,* 1988). Responsible for this pattern of motility are roughly symmetrical flagellar waveforms of relatively low amplitude. First observed in the laboratory by Yanagimachi during his work on hamster sperm (Yanagimachi, 1970), the motility pattern 'hyper-activation' has since been noted in all Eutherian mammalian sperm examined, including humans (Burkman, 1984). Hyperactivation involves the generation of high amplitude asymmetrical bends in the proximal midpiece, propagation of which along the length of the flagellum results in vigorous tail movements and unequal waveforms (Mortimer, 1997). The result in mammalian sperm is a reduced linearity of the sperm trajectory, but an increase in thrust (Suarez *et al.,* 1991).

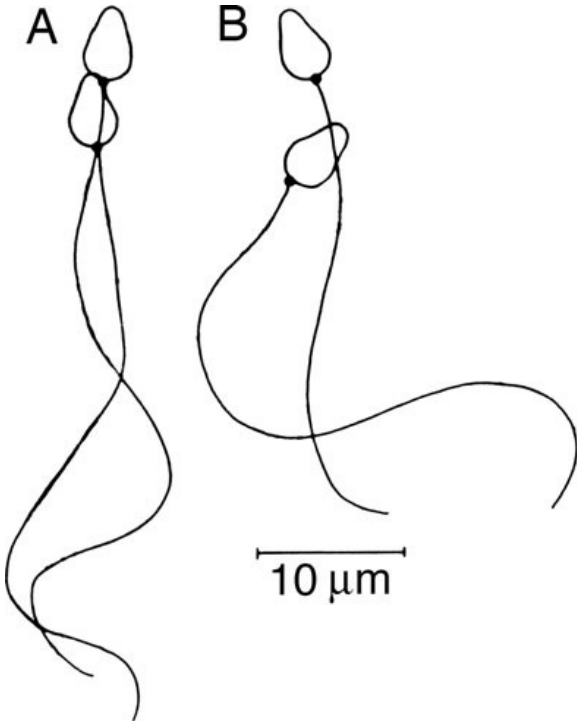

Figure 1.4: Beat pattern in activated vs hyperactivation sperm. 'A' = progressive sperm, 'B'= hyperactivated sperm. Image from (Suarez, 2008).

A reversible phenomenon, hyperactivation becomes initiated in the oviduct of mammalian species (Katz *et al.*, 1989; Mortimer and Swan, 1995; Suarez and Pacey, 2006). Rabbit sperm recovered from the ampulla were observed to swim in circles, a characteristic trajectory produced by asymmetric bending (Suarez *et al.,* 1983). In the hamster, hyperactivated sperm have even been observed 'in situ' through the transparent wall of the oviduct (Katz and Yanagimachi, 1980). The pH and ionic environment of the oviduct are supportive of hyperactivated motion, and follicular fluid has been shown to promote its expression (Fabbri *et al.,* 1998). The propulsion generated by this whiplash-like tail movement has been associated with sperm fertilising potential, both in vivo and in vitro, in animals (Ren *et al.,* 2001; McPartlin *et al.,* 2009), and in humans (Johnston *et al.*, 1994; Sukcharoen *et al.*, 1995).

Removal of sperm from seminal plasma, in which 'decapacitation' factors actively prevent premature sperm maturation (Chang, 1957), and re-suspension in a medium containing the necessary biochemical requirements, allows hyperactivation to be observed in vitro. Interestingly, hyperactivation in semen is actually associated with raised levels of the superoxide ion (de Lamirande and Gagnon, 1993), rendering its ready expression a possible negative factor under these conditions. Hyperactivation in vitro is supported when sperm are suspended in a medium containing at least 25mM bicarbonate, excess exogenous calcium ions and an energy source such as glucose. Other factors present in culture media may also support hyperactivation, such as taurine and a protein source such as human serum albumin or bovine serum albumin (Mortimer, 1997).

When free swimming human sperm are suspended in a low viscosity medium designed to support capacitation, two distinct patterns of hyperactivation can be observed. The first is a swimming style in which cells move generally forward, but via the production of very wide lateral displacements of the sperm head. This is referred to by some investigators as 'transition phase' (Mortimer and Swan, 1995). The second category of hyperactivation is a step up from this; movements are even more dramatic, and produce patterns described as 'figure-of-eight,' 'thrashing,' 'helical' or 'star-spin' (see Figure 1.5). In this case tail beating is so asymmetrical that cells move on the spot rather than in a space gaining manner (Robertson *et al.*, 1988; Mbizvo *et al.*, 1990; Mortimer and Swan, 1995).

If sperm are exposed to a medium of high viscosity, the amplitude of flagellar bending is automatically reduced (Suarez and Dai, 1992; Mohri, 1993). Hyperactivated movement in fact becomes translated into forward progression, presumably because much of force of the thrust has been absorbed. For example, when sperm cells enter the cumulus, their

movement changes in response to the viscoelasticity of the matrix; beat frequency and flagellar curvature are reduced, and sperm move progressively, albeit slowly (Mortimer, 1997). Sustaining hyperactivated movement requires increased energy production by the cell (Luconi and Baldi, 2003). Importantly, the extra ATP is used specifically to enhance flagellar curvature and not to increase tail beat frequency. The frequency of flagellar beating is actually reduced in hyperactivated cells (Mortimer and Mortimer, 1990).

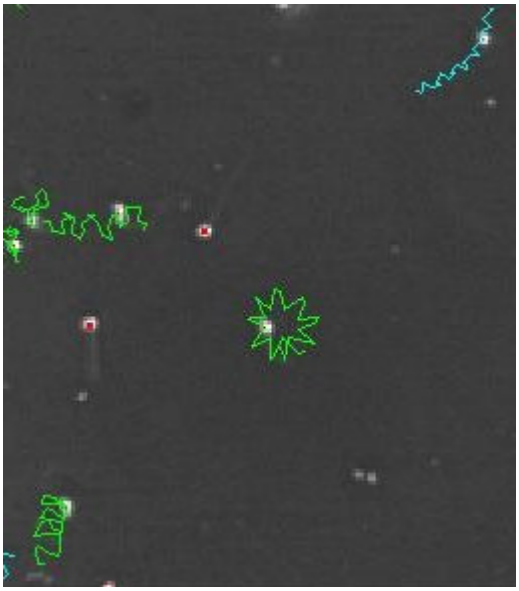

Figure 1.5: Human sperm in 'star spin'. Observed using Computer Assisted Sperm Analysis during an experiment carried out in Chapter 4.

1.5 Capacitation

Freshly ejaculated mammalian sperm are unable to fertilise eggs immediately, and instead require a prior incubation period in the female reproductive tract (Austin, 1951; Chang, 1951). To become 'capacitated' (Austin, 1952) sperm cells undergo a highly complex process of signal transduction, culminating in biochemical, molecular, physiological and structural alterations that render them fully mature and capable of interaction with an oocyte (De Jonge and Barratt, 2006). Capacitation is temperature dependent, occurs over a

period of hours, and is a reversible process. In the laboratory it can be induced with the use of a medium designed to mimic the oviductal lumen (De Jonge, 2005). Hyperactivated motion is usually considered a concomitant of capacitation, and the expression of hyperactivation may serve as a visual marker by which to identify a capacitation-supportive medium. However, whilst the two processes are certainly linked temporally, it is unclear to what degree their signalling pathways are shared. Plasma membrane changes brought about by capacitation may increase cell permeability to ions involved in controlling motility, and tyrosine phoshorylation of axonemal proteins by PKA, a downstream event of capactitation, may impart the flexibility required for increased flagellar bending (Mariappa *et al.*, 2010). However, there is evidence, both in the bovine and the human, that hyperactivation can occur completely independently of capacitation, suggesting that they are regulated separately, or at least that the signalling pathways diverge at some point (Ho and Suarez, 2001; Marquez and Suarez, 2004; Bedu-Addo *et al.*, 2008).

1.6 Function of hyperactivation

Hyperactivation has been associated with sperm fertilising potential both in vivo and in vitro, and in animals and humans. Four primary roles for hyperactivation have been postulated, as outlined below:

1. *Effective passage through viscous oviductal mucous*

   Experiments in the hamster demonstrated that hyperactivated cells pass more efficiently through viscous medium than those which are not (Suarez *et al.,* 1991). The lumen of the oviduct in mammals is filled with viscous fluid and mucus secretions (Jansen, 1980), and hyperactivation may facilitate movement through such secretions. Whilst the cervix also contains mucus, hyperactivation is unnecessary at this point,

possibly because the mucus is thinner, or because pressure changes assist sperm progress at this stage of the tract (Suarez and Dai, 1992).

2. Prevention from becoming trapped by epithelial wall of oviduct

Circular trajectories resulting from hyperactivation may allow sperm to change direction and prevent them from becoming trapped in the crypts which line the epithelial wall of the tubes, allowing easier progression towards the oocyte. Interestingly, human sperm in vitro have also been observed to seemingly deliberately bind to the endosalpingeal epithelium (Pacey *et al.,* 1995; Pacey *et al.,* 1995; Baillie *et al.,* 1997). Functionally this may represent a mechanism by which to hold sperm in a senescent state and preserve their motility, for example if insemination has preceded ovulation (Bjorndahl *et al.*, 2010). Another reasonable explanation for this phenomenon may be that, by letting sperm reach the ampulla in only small numbers at a time, the chances of polyspermic fertilisation are reduced. Sperm must of course be able to free themselves from these interactions at the appropriate time, and a putative method of accomplishing this is via the generation of deep and forceful thrusts, i.e. by hyperactivating (Suarez, 2008). In the mouse, only hyperactived sperm were observed to detach from the oviductal epithelium (Demott and Suarez, 1992). Similarly, sperm from mice which completely lacked the biological capacity to induce hyperactivation were unable to progress beyond the oviductal sperm reservoir in vivo (Ho *et al.,* 2009).

3. Infiltration of the cumulus oophorus

During the antral phase of follicular growth a dense layer of granulosa cells surrounds the oocyte and supports its development within the follicle. These cells persist after ovulation, and together with hyaluronic acid linked by proteins, make up the huge

cumulus oophorus matrix (Johnson, 2007). Penetration of this elastic meshwork seems to require the application of specific mechanisms by sperm cells. For example, sperm surface-bound hyaluronidase enzymes may degrade hyaluronic acid (Kim *et al.,* 2008), and the sheer mechanical force imparted by hyperactivated motility may also confer an advantage. In the mouse, it was demonstrated that hyperactivated sperm moved through medium containing polyacrylamide (which is of a similar viscoelasticity to the cumulus matrix) with greater speed and linearity than activated sperm (Suarez and Dai, 1992).

4. *Penetration of the zona pellucida*

The protective outer coat of the oocyte, composed of glycoproteins, presents the final barrier that sperm must overcome to reach the oolemma. Specific binding of capacitated sperm heads to the surface of the zona pellucida causes the proteolytic contents of the acrosomal store to be exocytosed (Johnson, 2007). These enzymes may have a role in dissolving a path through the zona pellucida (Florman *et al.,* 2008). However, the significance of this lytic function is debated. In mammals a proportion of sperm will release their acrosomal contents prior to even reaching the surface of the egg (Bedford, 1998), and in the mouse, sperm entirely deficient of acrosin can nonetheless breach the zona and fertilise eggs (Baba *et al.,* 1994). Mathematical calculations have also shown that the force generated by non-hyperactivated sperm would be insufficient to break the bonds between the sperm head and the zona proteins, and that hyperactivation would provide the necessary mechanical power for the sperm to wriggle or drill through the zona (Green and Purves, 1984; Patrizio *et al.*, 2000). The most conclusive evidence for the role of hyperactivation as the predominant mechanism of zona penetration comes from studies in the hamster and the mouse.

When hyperactivation was abolished, either by manipulating the culture medium or by creating knockout mice for a gene which is essential for this type of movement, in vitro fertilisation of zona-intact oocytes was completely prevented (Stauss *et al.,* 1995; Ren *et al.,* 2001). Similarly, in the equine, only sperm samples which hyperactivated in response to an inducer were able to fertilise oocytes in vitro (McPartlin *et al.*, 2009).

1.7 Chemotaxis

Sperm from marine invertebrates use chemotaxis to locate oocytes and fertilise them externally (Eisenbach, 1999). A potential role for this type of chemical guiding mechanism also exists in mammals, and a number of studies have reported an attraction of sperm to the oocyte-cumulus complex and follicular fluid (Ralt *et al.,* 1991; Wang *et al.,* 2001; Sun *et al.,* 2005). The aromatic aldehyde bourgenal has been shown to activate olfactory receptors found in human sperm, and to induce chemotaxis by influencing the generation of cAMP, which in turn may open Ca^{2+} channels and modify flagellar movement (Spehr *et al.,* 2004). An equivalent endogenous stimulant has not been identified. Progesterone is, in fact, the most likely candidate molecule for inducing this change in human sperm behaviour in vivo (Villanueva-Diaz *et al.*, 1995; Teves *et al.*, 2006; Gakamsky *et al.*, 2009). A tentative model for the molecular signalling pathway underlying the chemotactic response of mammalian sperm involves both the activation of adenylate cyclase and guanylate cyclase, followed by tyrosine phosphorylation of flagellar proteins and mobilisation/influx of Ca^{2+} (Teves *et al.,* 2009). It is still disputed, however, whether this ability of progesterone to draw sperm towards the oocyte is representative of specific movement along a concentration gradient (true chemotaxis), or non-specific changes of direction caused simply by induction of hyperactivation. The latter effect is chemokinesis, not chemotaxis,

however it would nonetheless hold the sperm in one place and allow them to 'search' for the cumulus (Jaiswal *et al.,* 1999).

1.8 Ca^{2+} signalling in sperm

Mature sperm cells are compact and highly motile. In order to achieve this status they are forced to sacrifice much of their cytoplasmic volume and condense their nucleus. With largely inaccessible DNA and no endoplasmic reticulum, sperm are thought to be transcriptionally and translationally silent (or at least very quiet) (Costello *et al.,* 2009). To therefore respond to external signals they rely heavily on post-translational modification of proteins via second messengers, the most crucial of which is intracellular calcium ion concentration, $[Ca^{2+}]_i$ (Publicover *et al.,* 2007). Aberrant Ca^{2+} signalling may contribute to impaired function in terms of cell maturation, acrosome reaction and hyperactivation (Evans and Florman, 2002; Kirkman-Brown *et al.*, 2002; Suarez and Ho, 2003).

1.9 Role of Ca^{2+} in generation of hyperactivation

A comprehensive overview of the signalling pathway is yet to be elucidated, but cytosolic Ca^{2+} concentration in the vicinity of the midpiece and tail is known to be the critical regulator of hyperactivated motility in mammalian sperm (Ho *et al.*, 2002; Harper *et al.*, 2004; Suarez, 2008). Demembranated-reactivated bull sperm models have shown that Ca^{2+} acts directly on cytoskeletal components of the axoneme (Ho *et al.,* 2002), binding to calmodulin or calmodulin-binding proteins to modify proteins which influence curvature (Ignotz and Suarez, 2005). The extra energy required for hyperactivation is derived from an increased consumption of ATP by axonemal components (Ho *et al.,* 2002).

Ca^{2+} for hyperactivation can be sourced from the extracellular environment, and it will move readily into the cell down its electrochemical gradient. A variety of Ca^{2+}-permeable channel families have been identified in the sperm flagellum, which in both mice and humans include classical voltage-gated, cyclic nucleotide-gated and transient receptor potential. Each could potentially play a role in the regulation of sperm movement and hyperactivation (Darszon *et al.*, 2006; Suarez, 2008). The family of genes which code for the proteins of the 'CatSper' channels, however, are the only plasma membrane channels that have been identified as fundamental for the generation of appropriate sperm movement, and in particular the expression of hyperactivation (Ren *et al.,* 2001).

1.10 CatSpers

Genes belonging to the CatSper family, discovered in the last decade through studies in knockout mice, are expressed exclusively in the testes and have been found to play a critical role in mammalian male fertility (Quill *et al.,* 2001; Ren *et al.,* 2001; Carlson *et al.,* 2003). Mice which are null for CatSper1, 2, 3 or 4 are healthy, undergo normal spermatogenesis and produce standard concentrations of swimming sperm, but are infertile (Ren *et al.,* 2001; Jin *et al.,* 2005; Jin *et al.,* 2007; Qi *et al.,* 2007). Only in vitro and with the prior removal of zona pellucidae can sperm from CatSper null mice fertilise eggs (Ren *et al.,* 2001).

The gene products of the CatSpers contain six-transmembrane-spanning regions and are thought to form a tetrameric cation channel which localises to the plasma membrane at the principal piece of the sperm tail (Kirichok *et al.,* 2006; Qi *et al.,* 2007). Patch clamping studies in human sperm have recently revealed that progesterone, secreted by the cumulus cells surrounding the egg, facilitates the opening of these voltage-sensitive and

alkaline-dependent CatSpers, either through direct binding to the channel itself, or via a subunit (Lishko *et al.,* 2011; Strunker *et al.,* 2011).

Abrogation of $I_{CatSper}$, the current which flows through the channel, disrupts sperm motility (Carlson *et al.,* 2003). Sperm from CatSper null mice are motile upon ejaculation, but this motility is unsustainable. Crucially, the ability of the tail to beat and bend more forcefully, and therefore also the initiation and maintenance of hyperactivatation, is lost completely (Ren *et al.,* 2001; Carlson *et al.,* 2003; Qi *et al.,* 2007).

CatSper studies have also been carried out in humans, although these are limited. Investigations conducted on the genetics of brothers, apparently with consanguinity in their family, revealed a human CATSPER2 homologue at loci 15q. The inheritance of two mutated copies of this autosomal gene resulted in non-syndromic male infertility (Avidan *et al.,* 2003). These men produced semen which was normal with respect to volume and sperm concentration, but was asthenoteratozoospermic. Less than 3% overall motility was observed, and sperm tails were short and coiled. In 2009, mutations were also identified mutations in the CATSPER1 loci (chromosome 11) in humans, again via studying men from consanguineous families presenting with infertility (Avenarius *et al.,* 2009). These men, however, as well as having asthenoteratozoospermia, also produced low volume ejaculates with low sperm concentrations, suggesting that in this case either disruption of motility may not be the sole role of human CATSPER1, or there were other underlying reasons for the suboptimal spermatogenesis. Another study, which analysed transcripts of CatSper from testicular tissue of men undergoing surgery either for a testicular biopsy or orchidectomy, found significantly lower levels of CatSper gene

expression in men for whom a motility disorder was causing them to be infertile (Nikpoor *et al.,* 2004).

1.11 Intracellular Ca^{2+} stores and hyperactivation

In somatic cells, which use changes in intracellular calcium ion concentration to control numerous biochemical processes, sophisticated temporal and spatial calcium signals can be generated in order to regulate complex Ca^{2+}-mediated processes (Berridge *et al.,* 2000). These cells make use of organelles such as the endoplasmic reticulum to store calcium ions internally, the subsequent release of which helps to active discrete responses (Michelangeli *et al.,* 2005). Sperm in comparison are cytoplasmically very simple, yet they can also generate distinct patterns of Ca^{2+}signals in different regions of the cell, bestowing on it a degree of compartmentalisation. For example, although both mechanisms involve a rise in Ca^{2+}, hyperactivation and the acrosome reaction can occur independently in both hamster and human sperm (Suarez and Dai, 1995; Harper *et al.*, 2004).

Whilst sperm lack endoplasmic reticulum, they are still able to store calcium ions. The acrosomal store, that is the acrosome itself, has been well characterised in both mice and humans (Florman *et al.,* 1998; Herrick *et al.,* 2005; Bedu-Addo *et al.,* 2007). However, more recent evidence has pointed to the existence of an additional storage organelle responsible for accumulating calcium ions at the neck/midpiece region of mammalian sperm.

In bull sperm, the application of thapsigargin, which depletes internal Ca^{2+} stores via its inhibition of SERCA (an ATPase channel responsible for Ca^{2+} uptake), initiated a modification in flagellar bend amplitude and symmetry (Ho and Suarez, 2001; Ho and Suarez, 2003). An effect on flagellar bending was noted even when extracellular calcium

was unavailable, indicating that the additional cytosolic Ca^{2+} could only have originated from inside the cell (Ho and Suarez, 2001). In mice, knockouts which were null for Catsper1 and Catsper2 were able to induce hyperactivation upon application of the store mobilising agent thimerosal, although this was unsustainable (Marquez *et al.,* 2007).

Presenting human sperm with a logarithmic progesterone concentration gradient (0-3µM) caused a slow elevation of $[Ca^{2+}]^i$ in a sub-population of cells (Harper *et al.,* 2004; Bedu-Addo *et al.,* 2007). In some of these cells, Ca^{2+} oscillations were superimposed on top of this signal (Harper *et al.,* 2004; Kirkman-Brown *et al.,* 2004; Harper *et al.,* 2005). Occurring specifically at the neck/midpiece region, the peaks of these oscillations coincided with periods of enhanced flagellar excursion, and the troughs with a subsequent relaxation (Harper *et al.,* 2004; Bedu-Addo *et al.,* 2008; Machado-Oliveira *et al.,* 2008). In a similar scenario to that described above in the bull, Ca^{2+} oscillations in human sperm were unaffected by the depletion of extracellular Ca^{2+}, but sensitive to EGTA, an agent known to chelate cytoplasmic calcium ions (Kirkman-Brown *et al.,* 2004; Machado-Oliveira *et al.,* 2008). The oscillations were proposed to represent cyclic mobilisation and re-filling of the Ca^{2+} store (Harper *et al.*, 2004).

Known to induce Ca^{2+} store mobilisation in other cells, when 2mM 4-AP was added to human sperm cells it also appeared to mobilise the store at the neck/midpeice (Grimaldi *et al.*, 2001; Gu *et al.*, 2004; Bedu-Addo *et al.*, 2008). Moreover, the rise in Ca^{2+} that this induced corresponded with a tenfold increase in hyperactivation in a population of cells, indicating that 4-AP may be the most powerful inducer of hyperactivation yet discovered.

Pharmacological manipulations of human sperm have indicated that the ryanodine-receptor may be responsible for gating the store (Harper *et al.,* 2004), pointing to a

mechanism of calcium-induced calcium release (CICR) (Harper *et al.,* 2004). Conversely, in bull sperm, store opening appears to be regulated by IP_3 and store-localised IP_3 receptors (Ho and Suarez, 2001). The redundant nuclear envelope is the structure most likely to be responsible for accumulating the calcium ions, and indeed the storage organelle secretory-pathway Ca^{2+}-ATPase (SPCA) has been localised to this region of human sperm (Harper *et al.,* 2005).

Recent work in human sperm has shown that some of the components required for Ca^{2+} signalling via the neck/midpiece store may be acquired by human sperm via prostasomes (Park *et al.*, 2011; Ren, 2011). Prostasomes are very small, membranous, prostate-derived vesicles which are ejaculated along with sperm. Interaction between prostasomes and sperm is potentiated by an acidic environment, so when they become mixed together in the vagina, prostasomes can fuse with the sperm plasma membrane and deliver to the cell proteins which promote ryandodine-receptor activation, the ryanodine receptor itself, and SPCA1. Fusion was concentrated around the midpiece of the sperm (Park *et al.*, 2011), perhaps with a view to arming the Ca^{2+} store in that location. This novel discovery will lead to further exploration of the putative role of aberrant prostasome-sperm fusion with deficiencies in motility.

Therefore, whilst its identity and precise mode of operation are as yet unclear, since flageller beating is initiated at the anterior part of the sperm tail, a midpiece store is ideally placed to provide calcium ions to the axoneme for the modulation of flagellar bending. Sperm have the ability to rapidly switch between hyperactivated and activated motility, for example to ‘burrow’ through the zona pellucida (Mortimer and Swan, 1995; Bedford, 1998). The mobilisation of stored calcium ions represents an elegant method by which

sperm could regulate such events, and enable quick and reversible bouts of hyperactivated motility.

In conclusion, it seems that a model for the induction, maintenance and modulation of hyperactivation involves the combination of both Ca^{2+} influx at the plasma membrane, primarily from CatSpers, plus Ca^{2+}-release from an internal store. Recent observations in mouse studies have generated the interesting hypothesis that CatSper activation may produce bending in one direction, through phosphorylation of tail proteins, whereas store-mobilisation may reverse this bending via dephosphorylation. If such activities were to enable direction changes in the female reproductive tract, then it may be that CatSpers and storage organelles are directly involved in navigation and/or chemotaxis (Chang and Suarez, 2011).

Chapter 2: Introduction to the assessment of hyperactivation for clinical purposes

2.1 Infertility in men

Male factor infertility is a significant, and, it has been suggested, increasing global problem (Sharpe and Irvine, 2004). Aberrant spermatogenesis and/or inadequate sperm function is implicated in around 40% of subfertility cases, either in isolation, or in conjunction with a female component (Simon *et al.*, 2010).

2.2 Asthenozoospermia

Inadequate generation of motility, termed asthenozoospermia, can cause infertility in males. A retrospective, large-population study on motility related subfertility revealed that some degree of motility problem afflicted approximately 80% of infertile men (Curi *et al.*, 2003). Approximately 20% of men in the study had asthenozoospermia in isolation, i.e. no other sperm defects were apparent at semen analysis (Curi *et al.,* 2003). Severe motility dysfunction may be caused by defective flagellar architecture, either through non-specific structural anomalies or congenital diseases causing ciliary dyskinesia, such as Kartagener's syndrome (Chemes *et al.*, 1998; Chemes, 2000). Usually responsible for less pronounced forms of asthenozoospermia, membrane damage by reactive oxygen species and inefficient operation of signalling pathways can also impact negatively on sperm motion (Calamera *et al.,* 2003). Whilst there is limited data to show that drugs such as L-acetyl-carnitine, and even pure FSH (Acosta *et al.*, 1992), can improve motility parameters in patients presenting with idiopathic asthenozoospermia (Balercia *et al.,* 2005), by far the most effective and most employed treatment for most types of male infertility is assisted reproductive technology.

2.3 Semen analysis

Pathologies of sperm motility are largely diagnosed during a standard, 'descriptive' laboratory semen analysis. A basic analysis of seminal fluid will usually comprise a macroscopic examination of the ejaculate, which indicates if semen production is being carried out normally, followed by a microscopic examination of all the cells, in particular of course the sperm. The values for sperm concentration, percentage motility and percentage normal morphology are then compared against reference criteria endorsed by the World Health Organisation (WHO, 2010). With respect to motility, the focus is on the absolute number, or the proportion, of forward progressive sperm in semen, which has been shown to be an important determinant of fertility in vivo (Barratt *et al.*, 1992). Specifically, the number of sperm moving with high linear velocity, whilst simultaneously exhibiting moderate lateral deviation of the sperm head, has been associated with the ability of a sample to colonise the cervix following ejaculation and migrate through cervical mucus (Aitken *et al.,* 1986; Mortimer *et al.,* 1986). Such a measure, however, provides no direct insight into how sperm will move in the upper part of the female reproductive tract, that is, once they have been exposed to capacitating conditions and are faced with the task of navigating the oviduct and penetrating egg vestments. Indeed, the signalling pathways involved in generating 'activated' versus 'hyperactivated' motility (discussed in detail in Chapter 1) are distinct.

Whilst a basic semen analysis is the cornerstone of male fertility investigations, and is adept at identifying men whose chances of fertilising oocytes is very low, for example those with severe oligo-, astheno-, or teratozoospermia, with respect to the majority of cases, semen analysis does not have the power to unambiguously predict the chances of spontaneous conception, or indeed success with a particular form of assisted reproduction

(Tomlinson *et al.*, 1999). Even when confounding female factors are removed, and analyses are carried out to a high degree of accuracy, researchers have highlighted the fact that there is considerable overlap between the semen parameters of fertile and infertile men (Guzick *et al.,* 2001; Jedrzejczak *et al.,* 2008). Moreover, the fact that data gathered by the HFEA suggests that up to one quarter of the UK's subfertile couples are diagnosed with 'unexplained' infertility, it seems entirely plausible that unidentified or 'hidden' male factor defects could be contributing to this statistic.

2.4 Sperm function testing

Extended semen analysis and sperm function testing may hold the key to identifying a greater number of male factor disorders (Oehninger, 2000). By assessing, either directly or indirectly, more sophisticated functional aspects necessary for both passage through the female tract and oocyte interaction, a clearer prognosis with respect to a sperm sample's fertilising potential may be achieved. Such methods were developed prior to the widespread use of ICSI, in a climate geared towards the examination of sperm defects and the determination of their etiology, with a view to finding ways to enhance sperm performance.

Particular interest was directed towards gamete interaction, for example, the ability of sperm to bind to the zona pellucida or acrosome react (Burkman *et al.,* 1988; Franken *et al.,* 1989; Cummins *et al.,* 1991). A meta-analysis of such bioassays demonstrated that their predictive power with respect to fertilisation outcome in IVF was indeed high (Oehninger *et al.,* 2000).

2.5 CASA

The development of effective computer-assisted sperm analysis, which provided a digital, automated means by which to measure cells in real-time, evoked enthusiasm for the study of sperm motility characteristics and their influences on fertilising ability. CASA has two distinct advantages over manual motility assessment by eye, namely that it can determine cell velocity with a high degree of accuracy and that it can assess literally hundreds of cells in a matter of seconds. Whilst some studies have indicated that CASA may be as reliable as manual methods for carrying out some aspects of semen analysis (Tomlinson *et al.*, 2011), its key strength lies in its ability to measure extremely subtle kinematic parameters, for example straightness of sperm swimming and lateral displacement of the sperm head. Obtaining numerical values for these would be impossible to accomplish by eye, and highly time consuming using laborious older methods such as videomicrography combined with manual trajectory reconstruction (Mortimer, 1997).

CASA with negative phase contrast optics identifies human sperm in a field of view based on settings pertaining to their size and illumination (Mortimer, 1997). By tracking each sperm head and reconstructing its trajectory, a wealth of mathematical information can be gained regarding particular aspects of sperm motion. Using the centroid (the centremost point of the head) as a reference point, the primary kinematic values determined are those of three distinct velocities (Figure 2.1). Each refers to the distance travelled in within a set time period, and is therefore a direct reflection of velocity. The curvilinear velocity (VCL) is a measure of the overall distance travelled in any direction. It is dependent on the frequency of tail beating, and the wavelength and amplitude of the flagellar movement (Suarez and Dai, 1992). The straight-line velocity (VSL) is the distance between the start and end of the sperm trajectory, and it therefore describes the distance covered in a straight

line. Average path velocity (VAP) is the length of the general trajectory, that is, the distance the sperm travels in the average direction of its movement. It is calculated by the CASA using sophisticated 'smoothing' methods.

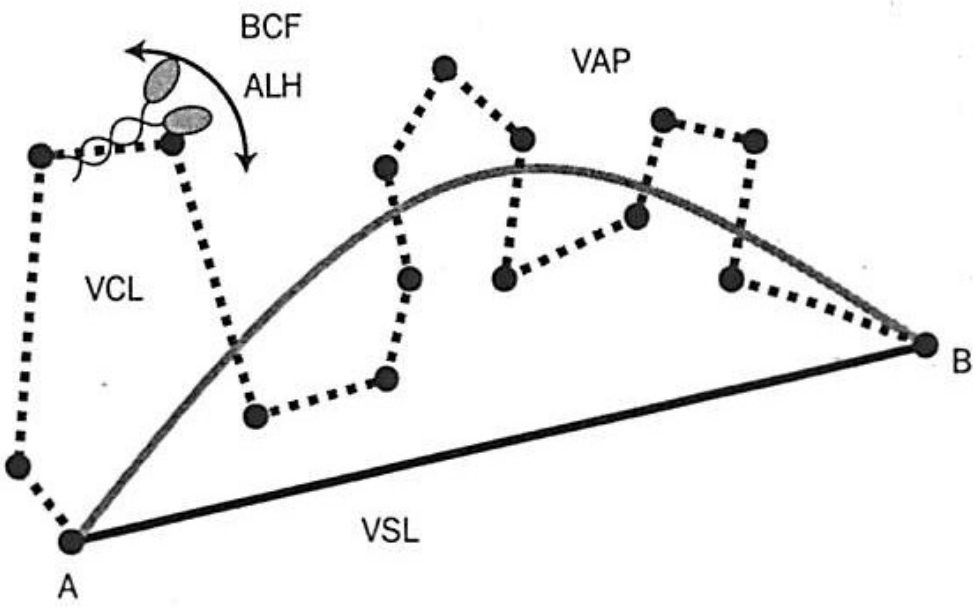

Figure 2.1: Kinematic parameters measured by CASA. Schematic showing a digitally reconstructed sperm trajectory and the kinematic values recorded. From (Oehninger and Kruger, 2007).

From these velocities, additional numerical values have been derived, namely, linearity (LIN), which equals (VSL/VCL) x 100, and straightness (STR), which is (VSL/VAP) x 100. These provide comparative information regarding the different paths observed, allowing greater distinction between the types of motility, be it progressive or otherwise.

The amplitude of lateral head displacement (ALH) measures the width the sperm head moves from side to side, and to an extent corresponds to the degree of bending initiated at the sperm neck (Oehninger and Kruger, 2007). Calculations of its value are based upon the

distance between the peaks and the troughs of the centroid's path, and often this value is expressed as the mean of a series of values measured all along the trajectory (HamiltonThorne). The derivation of beat/cross frequency (BCF) works on the assumption that with each new flagellar beat the curvilinear path will cross the average path, and this is how it is calculated (Mortimer, 1997).

2.6 Assessment of hyperactivation using CASA

CASA has enabled the direct assessment of hyperactivation levels in a sperm sample, which doing by eye would be uncontrolled, subjective, and extremely difficult. Hyperactivation is of course a flagellar phenomenon, but the waves themselves are extremely difficult to measure due to the frequency and subtleties of their movement. A highly time-consuming process, only a relatively low proportion of sperm can be analysed (Suarez, 2008). Therefore, CASA systems track the sperm head. Whilst this is only an indirect measurement of the tail movement, it has been validated as a satisfactory method of hyperactivated cell identification (Mortimer, 1997).

The transition to hyperactivated movement in human sperm involves an increase in overall cell speed combined with more pronounced, asymmetric, flagellar bending. These changes are directly reflected in sperm trajectories; VCL and ALH increase, whilst LIN correspondingly is reduced. Algorithms used to define a hyperactivated cell are conventionally based around threshold values for VCL, ALH and LIN levels, allowing the specific isolation of cells comprising meeting requirements for all three. It can then be established what proportion of the total number of cells are moving in this manner (i.e. the % hyperactivation). This has been found to be a more appropriate measure of the degree

of hyperactivation in a sample than using population averaged values for VCL, ALH and LIN (ESHRE, 1996).

2.7 CASA hyperactivation screens for clinical use

Numerous investigators have attempted to address whether or not CASA measurements of kinematic parameters in semen could be useful in providing prospective indications regarding sperm fertilising capacity, both in vivo and in vitro, with some yielding encouraging results (Barratt *et al.*, 1993; Paston *et al.*, 1994; Macleod and Irvine, 1995; Lefievre *et al.*, 2009) Moreover, the expression of hyperactivation in an appropriate medium, a parameter which could not be assessed using routine andrology diagnostics, yet which had potential clinical significance, became a particular focus of investigation (Sukcharoen *et al.,* 1995; Sukcharoen *et al.,* 1995; Chan *et al.,* 1998).

Intriguing results emerged from clinical studies of hyperactivation under IVF conditions. IVF using conventional insemination presents an ideal system with which to measure the effect of this movement pattern on overall sperm function. Whilst the female reproductive tract is discounted, the zona and cumulus (passage through which is, arguably, the primary requirement of hyperactivation) remain. By using fertilisation as an end point with which to judge sperm competence, instead of, for example, pregnancy in vivo, an in vitro scenario is influenced by fewer variables and confounding factors. Investigators reported correlations between hyperactivation rate and fertilisation rate, and degrees of positive and negative predictive power (Wang *et al.,* 1993; Guerin *et al.,* 1995; Sukcharoen *et al.,* 1995; Chan *et al.,* 1998). Interestingly, one study even went so far as to use in vitro fertilisation outcome as the functional end point by which to define the criteria that best identified a

hyperactivated subpopulation of cells (Sukcharoen *et al.,* 1995), suggesting that hyperactivation was a truly critical functional parameter which should not be ignored.

2.8 Problems surrounding assay development

Whilst evidence suggested that not only did hyperactivation-specific pathologies exist, but that their identification may be important in clinical decision making, a lack of standardised, uniform criteria for its measurement produced results which were essentially non-transferrable to other laboratories (Oehninger *et al.,* 2000).

For example, significant confusion surrounded which specific movement characteristics (as quantified by CASA) actually denoted a hyperactivated cell, and no consensus was established on this. Different authors proposed, and utilised, different algorithms for this task (Robertson *et al.*, 1988; Grunert *et al.*, 1990; Mortimer and Mortimer, 1990; Burkman, 1991; Zhu *et al.*, 1994; Sukcharoen *et al.*, 1995). The result of this was that particular algorithms may have held value within the context of the setting in which they were developed, but were not necessarily translational to other laboratories (ESHRE, 1996). As well as differences in opinion as to what type of movement specifically constituted hyperactivation, discrepancies between makes and models of CASAs, which operated using different frame rates and therefore did reconstruct like for like trajectories, served to further compound this problem (Zhu *et al.,* 1994). Disparities in sperm preparation techniques, media constituency and slide chamber depths also contributed to the generation of inconsistent, non-transferrable results (ESHRE, 1996; ESHRE, 1998).

Perhaps an even greater problem than the technical aspects posed by the measurement of hyperactivation, is the manner in which hyperactivation is actually displayed. For example,

human sperm populations differ from that of other animals in that only a relatively small proportion of cells display natural hyperactivation at any one time, that is, approximately 20% (Burkman, 1984). Distinguishing between 'low ' and 'high' hyperactivation levels is therefore difficult, and the opportunity for error if insufficient sperm are counted (i.e. only 100) is large (WHO, 1999), a detail some previous studies failed to acknowledge.

The expression of spontaneous hyperactivation has also been observed to vary over time (Sukcharoen *et al.,* 1995; Chan *et al.,* 1998), rendering it difficult to judge when exactly to conduct a CASA assessment and reliably evaluate hyperactivation. Furthermore, hyperactivated movement has been described by observers as 'biphasic,' meaning that cells can switch in and out of hyperactivation over a period of a few seconds and apparently at random (Mortimer and Swan, 1995). Indeed this behaviour is thought to be exaggerated in vitro (Suarez, 1996; Pacey *et al.*, 1997).

2.9 Assay development and modification

Many of these historical problems can now be addressed, however, and with appropriate use of equipment and technique optimisation it seems possible that hyperactivation assays have the potential to deliver reproducible and reliable results. For example, whilst in ideal circumstances, independent validation of algorithms used to denote a hyperactivated cell (using either visual methods or flagellar analysis) should be employed for each machine, ensuring the use of criteria previously validated for an identical sampling frequency and smoothing algorithm is likely to be sufficient to generate consistent results (Mortimer, 1997). In addition, the adherence to guidelines regarding CASA use would also help to improve and standardise results (ESHRE, 1998). Such guidelines recommend the use of only sperm preparation techniques which produce no deleterious effects on sperm function

(Mortimer, 1994), the use of a defined culture medium which does not enhance the generation of reactive oxygen species (Gomez and Aitken, 1996) and a slide chamber deep enough to permit sufficient flagellar excursion.

Stimulants known to elicit enhance hyperactivation, such as progesterone or pentoxifylline (Mbizvo *et al.,* 1993; Johnston *et al.,* 1994; Fabbri *et al.,* 1998) may enable better synchronisation of sperm populations. In theory what they measure is the full *potential* of cells to hyperactivate, rather than just their hyperactivation levels at one specific time point. Speculatively, this modification may both solve the problem of when to conduct a hyperactivation assay and/or go some way to counteract biphasic expression. One assay format involved a one hour pre-incubation of sperm samples with progesterone plus pentoxfylline. This assay demonstrated, quite impressively, 100% specificity and sensitivity in distinguishing poor (0-49%) from good (50-100%) fertilisation rates, however the sample size was small at only 29 cases (Bjorndahl *et al.*, 2010). The heating of sperm to 40^{o}C degrees has also been shown to enhance hyperactivation, and the ability to induce hyperactivation in response to heating linked to fertility potential (Chan *et al.,* 1998).

2.10 Development of a hyperactivation assay in the current era of assisted reproduction

Most of the investigative work into the clinical significance of hyperactivation was conducted in the early to mid 1990's, that is, before guidelines for obtaining accurate and transferrable results were laid down. The widespread use of ICSI thereafter prompted a decline in sperm function research, and the true potential of a hyperactivation screen was never really elucidated. However, a number of recent developments justify further exploration of the clinical significance of hyperactivation in human sperm; not least of which is the fact that animal studies have its fundamental biological role in fertilisation in a

number of species (Stauss *et al.*, 1995; Qi *et al.*, 2007; McPartlin *et al.*, 2009). The expression of hyperactivation by human sperm has also been clearly liked to other aspects of sperm function. For example, a positive correlation has been noted between the expression of hyperactivated motility in human sperm and the zona pellucida-induced acrosome reaction of zona-bound sperm (Liu *et al.*, 2007). With respect to sperm morphology, cells displaying hyperactivated motility under appropriate measurement conditions had a highly significantly increased chance of being morphologically normal than cells not exhibiting this movement pattern (Green and Fishel, 1999). Experiments assessing sperm chromatin have also linked the presence of high DNA damage with low levels of hyperactivation (Chan *et al.,* 2001). As the integrity of the paternal genome is proposed not only to impact on the incidence of fertilisation, but also to exert 'late' effects on embryo development (Teves *et al.,* 2006), there are significant negative implications associated with increased DNA fragmentation. All of these findings imply that the assessment of a sperm sample's ability to promote hyperactivation may not simply provide information about its mechanical capabilities in terms of penetration of the barriers posed by the latter part of the female reproductive tract and egg vestments, but that it may also serve as a biomarker by which to identify and an all-round good quality sample.

There exists also the potential to trial an entirely novel agonist in a hyperactivation assay. The ability of 4-AP to induce a dramatic increase in hyperactivation, which persists until the drug is removed (Gu *et al.*, 2004), may render it a more effective stimulant than those favoured previously. Once a reliable protocol was established, an assay of hyperactivation using 4-AP could be carried out very simply and quickly, in contrast to other tests of sperm function which involve more laborious procedures and the use of scarce biological material (Burkman *et al.,* 1988; Franken *et al.,* 1989).

The Assisted Conception Unit within Ninewells Hospital, Dundee, presents an excellent opportunity in which to study hyperactivation in the context of a clinical setting. With a relatively conservative use of ICSI (employed in less than 40% of all inseminations) and active patient recruitment for research, the clinic yields a number of ‘surplus’ samples from conventional IVF each week. By comparing hyperactivation levels in a sperm sample with its potential to fertilise oocytes in IVF, it may be possible to establish whether or not a hyperactivation screen has any merit as a prognostic tool in the current era of ART.

Chapter 3: Aims and objectives

A series of experiments were designed and carried out in order to accomplish four primary objectives, outlined below.

1. Selection of an agonist for use in a hyperactivation assay.
2. Exploration of the link between hyperactivation and subfertility.
3. Conduction of a study comparing IVF fertilisation outcome with hyperactivation levels.
4. Examination of the effects on hyperactivation of pharmacological agents known to interact with store-operated calcium signalling pathways.

Chapter 4: Experimental procedures

4.1 Media and reagents

Four different culture media were used across the experimental chapters. Clinical samples, and donor samples used for comparable analysis with clinical samples, were tested in Cook Medical Sperm Medium™ (capacitating, bicarbonate buffered) for IVF, and Cook Medical Gamete Buffer™ (non-capacitating, HEPES buffered) for ICSI. Experiments not involving clinical samples were conducted using laboratory synthesised corresponding media, for economic purposes (Tables 4.1 and 4.2). The components used were very similar to the commercial media, but HSA was replaced with BSA.

Capacitating Medium (CM)		
Component	*Sigma Cat. No*	*Final Concentration in dH_2O (mM)*
$CaCl_2$	C3306	3
KCl	P5405	4.7
$MgSO_4$, $7H_2O$	M2773	1
NaCl	S5886	106
$NaHCO_3$	S5761	25
NaH_2PO_4	S5012	1.5
D-glucose	G6152	5.6
Na pyruvate	P2256	1
Sodium lactate	L7900	41.8
Glycine	G7403	1.33
Glutamine	G3126	0.68
Taurine	T0625	0.07
Non-essential amino acids	M7145	0.01
BSA	A9418	30mg/ml

Table 4.1: Recipe for Capacitating Medium. CM was made up once per month from stock solutions and deionised H_2O. pH was adjusted to 7.4 using 1M NaOH, and osmolarity to 290-320 mOsm using NaCl. BSA was added only immediately prior to use media use, i.e. 30mg/ml each day. To eradicate microorganisms, media was then filtered using a 0.22µm diameter filter, and stored at 5^{o}C. Adapted from (Mortimer, 1986).

Non-capacitating Buffer (NCB)		
Component	*Sigma Cat. No*	*Final Concentration in dH_2O (mM)*
$CaCl_2$	C3306	1.8
KCl	P5405	5.4
$MgSO_4$, $7H_2O$	M2773	0.8
NaCl	S5886	116.4
NaH_2PO_4	S5012	1
D-glucose	G6152	5.6
Na pyruvate	P2256	2.7
Sodium lactate	L7900	41.75
HEPES	H3375	25
BSA	A9418	3mg/ml

Table 4.2: Non-capacitating Buffer. NCB was made up once per month from stock solutions and deionised H_2O. pH was adjusted to 7.4 using 1M NaOH, and osmolarity to 290-320 mOsm using NaCl. BSA was added only immediately prior to use media use, i.e. 3mg/ml each day. To eradicate microorganisms, media was then filtered using a 0.22µm diameter filter, and stored at 5^oC.

The pharmacological agents used in attempt to modify the expression of hyperactivation are detailed in Table 4.3.

Agent	Formula	Supplier	Catalogue no.
Progesterone	$C_{21}H_{30}O_2$	Sigma	P0130
4-aminopyridine (4-AP)	$C_5H_6N_2$	Sigma	275875
3-Isobutyl-1-methylxanthine (IBMX)	$C_{10}H_{14}N_4O_2$	Sigma	I7018
2-Aminoethoxydiphenylborate (2-ABP)	$C_{14}H_{16}BNO$	Calbiochem	100065
SKF-96365, Hydrochloride (SKF-96365)	$C_{22}H_{26}N_2O_3$. HCl	Calbiochem	567310

Table 4.3: Agents applied to sperm in an attempt to modify hyperactivation.

4.2 Procurement of sperm samples

Sperm samples were sourced both from a panel of research donors and from men attending the Assisted Conception Unit at Ninewells Hospital, Dundee, for IVF or ICSI. Patients undergoing IVF or ICSI with their partners were invited to participate in research by donating sperm samples that were surplus following oocyte insemination. Ethical approval was secured (Tayside Committee of Medical Research Ethics B, number 09/s1402/6), and informed consent was provided by everyone who took part (consent

form shown in section 10.2). Research donors were recruited and consented in accordance with HFEA regulations governing the donation of sperm for research.

4.3 Sample collection

Patient semen samples were produced by masturbation, following two to five days of sexual abstinence, on the day of their partner's oocyte retrieval. The ejaculates were subsequently placed a in 37^{o}C warming oven until full liquefaction was achieved, with processing commencing no later than one hour post production. Research donors were instructed to produce their samples in their home and then deliver them to the laboratory, whilst keeping them warm, within one hour. Upon arrival, sample processing began immediately.

4.4 Semen analysis

In the Assisted Conception Unit, semen analysis was performed manually by an embryologist. Concentration was determined using the dilution method and an Improved Neubauer Chamber. Motility was assessed at 37^{o}C, and grading was carried out using the A, B, C, D scoring system, detailed in (Bjorndahl *et al.*, 2010). Morphology assessment and an IgG MAR test for anti-sperm antibodies were conducted at a prior diagnostic appointment. Sample parameters were compared against criteria from (WHO, 1999) for normal limits, that is, concentration ≥ 20M/ml, motility ≥ 50% and morphology ≥15%. If semen analysis yielded a 'normal' result or pointed to a mild male factor component, then samples were scheduled for IVF. Indications for ICSI were analyses showing moderate to severe male factor components, the presence of clinically significant anti-sperm antibody binding, or a post-preparation recovery showing less than 1 million progressively motile sperm, less than

80% progressively motile sperm, or predominantly sluggish progressive motility. ICSI would also be favoured had a previous cycle resulted in a very poor fertilisation rate of MII eggs.

Research donor semen was analysed using CASA for concentration and motility. Sperm morphology and anti-sperm antibodies were not assessed. Donor samples were only included if they met WHO criteria for 'normal' semen in terms of concentration and motility (WHO, 1999). Semen samples with a round cell concentration of over 5M/ml were excluded from the study.

4.5 Sperm preparation

Clinical samples from men undergoing IVF or ICSI were prepared in the embryology laboratory using discontinuous density gradient centrifugation followed by a simple wash step. Briefly, 1ml of room temperature 80% Pure Sperm™ in HEPES-based Gamete Buffer™ was overlaid with 1ml of the corresponding 40% fraction, with care being taken to ensure that a clear interface was obtained. Up to 2.5ml of semen was then layered on top of this, with ejaculates greater than 2.5ml being distributed between two gradients. The gradient tubes were centrifuged for 20 minutes at 300xg. Following removal of all supernatant layers, pellets were then extracted and washed for 10 minutes at 500xg, either in 5ml Cook Sperm Medium™ (if IVF) or 5ml Cook Gamete Buffer™ (if ICSI). The final pellet/s was then resuspended in 1ml of the appropriate medium. ICSI samples in HEPES-based Gamete Buffer™ were stored at room temperature on the bench for approximately five to six hours until insemination. IVF samples in bicarbonate-buffered Sperm Medium™ were 'gassed' with CO_2 and stored at room temperature for approximately four to five hours, before being incubated for one hour at 37^oC and 6% CO_2 prior to insemination. An identical protocol was followed in the research laboratory for the processing of research donor

sperm, which was prepared in either Sperm Medium™ or Gamete Buffer™ depending on whether the sample was being compared with IVF or ICSI sperm. Procedurally, the only difference in handling of the research laboratory samples was that these were sometimes screened earlier than six hours post production; however, this was validated as an acceptable alteration (see Chapter 10.1).

4.6 Assessment of hyperactivation using CASA

A Hamilton Thorne 'CEROS' Computer Assisted Sperm Analyser was used for the assessment of sperm kinematics in all experiments of this nature. With negative phase contrast trinocular optics and a black and white CCD camera, this model was combined with Version 12 software, specifically the clinical (human) application.

The settings used in the screening program were kept constant, and are shown in Table 4.4.

Setting	Value
Magnification	10x objective (100x overall magnification)
Slide chamber depth	20microns
Frame rate	60 Hz
Number of frames recorded	30

Table 4.4: CASA settings.

A Hamilton Thorne 2X-CEL (20µm depth) microscope slide plus cover slip were pre-warmed for two minutes on a MiniTherm heated stage mounted on the CASA microscope. It was noted that recommendations designate the use of a 30µm chamber depth for the measurement of hyperactivation, to avoid any constraint in flagellar bending, however these were unavailable. In any case, the fact that many samples were still able to generate very high levels of hyperactivation suggests that this was unlikely to be a particularly limiting or distorting factor. Sample concentration was adjusted to 10-20 M/ml, and the

sample aspirated gently to ensure homogenous distribution of cells. 4µl of this sample was then pipetted onto each slide chamber of the pre-warmed slide, and covered carefully with the pre-warmed cover slip (see Chapter 10.1). CASA scanning was delayed for 30s to ensure full equilibration of sperm to 37^{o}C, and the cessation of any 'drift.' To obtain representative measurement a total of 800 motile cells were assessed (see Chapter 10.1). This was accomplished by scanning at least four microscope fields (at approximately 15M/ml this equates about 200 cells) in four slide chambers (Figure 4.1). The playback feature was used routinely to ensure that the majority of sperm cells were picked up, and that drift and the presence of round cells or debris were not biasing scan results. Where sperm were seen to be omitted from the analysis or round cells or debris seen to be included, the interactive quality control plot feature was used to readjust the size and intensity parameters used to define a sperm head in an attempt to capture as many sperm as possible. Once four lines of data, each comprising at least 200 motile cells, were obtained then the slide was removed from the heated stage and either washed (a maximum of four times altogether) or discarded. Tables 4.5 and 4.6 detail the motility parameters measured and recorded for each screen. Data was transferred from CASA to a specifically designed Excel sheet, and manipulated from there.

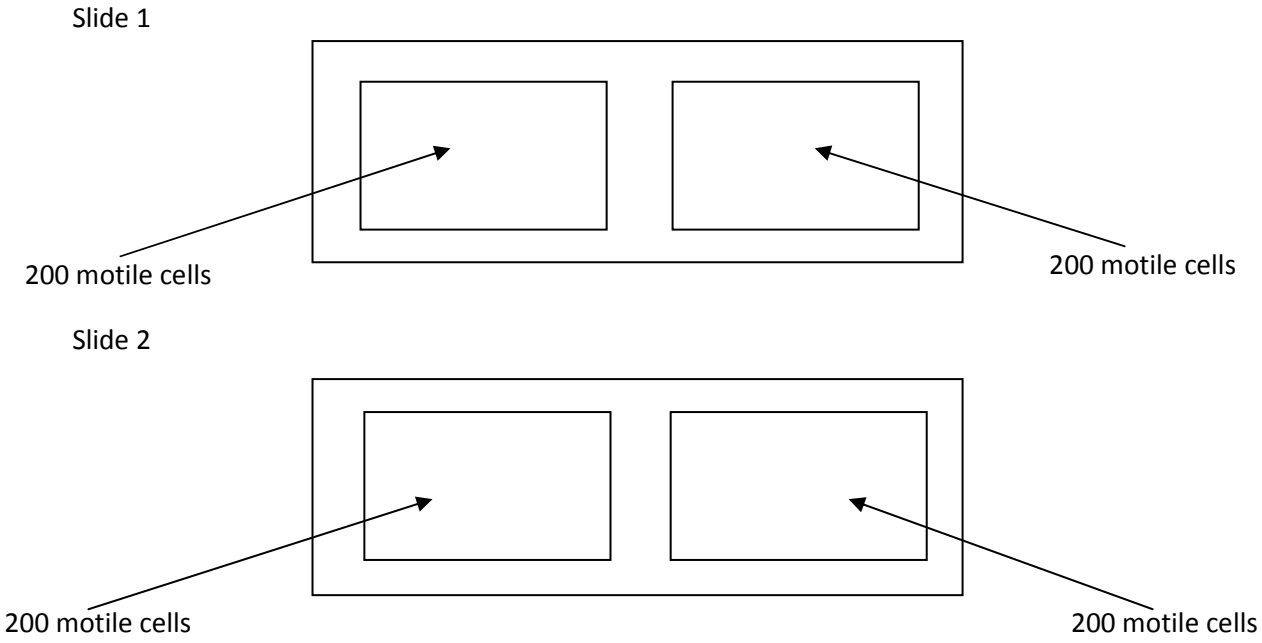

Figure 4.1: Method used for scanning microscope fields. Protocol was designed in an attempt to avoid bias introduced by 'problem' slide chambers.

Cell counts (number and/or %) plus concentration (M/ml)
Total
Motile (rapid plus medium)
Progressively motile (VAP □ 25 µm plus STR ≥ 80)
Rapid (VAP □ 25µm/s)
Medium (VAP 5-25µm/s)
Slow (VAP < 5µm/s plus VSL < 11 µm/s)
Static (immotile)
Hyperactivated* (VCL ≥ 150µm/s, ALH ≥ 7µm, LIN ≤ 50, ≥ 13 track points available (from possible 30))

Table 4.5: Classification of cells by CASA. *Algorithm validated for frame rate in (Mortimer *et al.,* 1998). Note: despite having a high VCL, hyperactivated cells moving on the spot (e.g. star spin) are often classified as 'slow' cells. Percentage hyperactivation was defined as the number of hyperactivated cells divided by the total number of *motile* cells.

Means plus bar chart distributions	**Units**
VAP	µm/s
VCL	µm/s
VSL	µm/s
ALH	µm
BCF	Hz
LIN	VSL/VCL
STR	VSL/VAP

Table 4.6: Kinematic parameters recorded by CASA.

Chapter 5: Selection of an agonist for use in a hyperactivation assay

5.1 Introduction

Support of capacitation and hyperactivation requires the suspension of sperm in fluids from the oviduct or a synthetic medium designed to mimic this environment (De Jonge, 2005). Hyperactivation is further enhanced by agents known to specifically promote its expression. Both compounds present in the female reproductive tract, such as progesterone and nitric oxide (Publicover *et al.,* 2007; Lefievre *et al.,* 2009), and a range of non-endogenous agents, can elicit hyperactivation in vitro for the purpose of analysis. Published work on hyperactivation stimulants indicate that these can be broadly divided into two categories: those that cause an elevation of intracellular calcium ion levels and those that influence the AC-cAMP-PKA pathway.

In mammals, examples of reagents which directly affect $[Ca^{2+}]_i$ include calcium ionophores (Suarez *et al.,* 1987), procaine, caffeine (Marquez and Suarez, 2004), thimerosal and thapsigargin (Ho and Suarez, 2001), 4-aminopyridine (Gu *et al.*, 2004; Chang and Suarez, 2011) and progesterone (Publicover *et al.,* 2007). The immediate elevation of cytosolic calcium ion concentration has been shown to correspond with an increase in flagellar bend and asymmetry (Blackmore *et al.*, 1990; Baldi *et al.*, 1991; Bedu-Addo *et al.*, 2007; Gakamsky *et al.*, 2009; Kilic *et al.*, 2009; Lishko *et al.*, 2011; Strunker *et al.*, 2011).

The primary stimulant of hyperactivation in vivo appears to be progesterone, and indeed this modification of motility appears to be the primary action of this steroid on sperm cells (Aitken *et al.*, 1996; Publicover *et al.*, 2007; Bedu-Addo *et al.*, 2008). Progesterone at nM-µM concentrations can also induce the acrosome reaction, and the ability of sperm to raise intracellular calcium ion levels in response to progesterone has been linked with

fertilisation potential in humans (Krausz *et al.,* 1995; Krausz *et al.,* 1996; Forti *et al.,* 1999; Giojalas *et al.,* 2004). As sperm are thought to be transcriptionally inert, in these cells the steroid hormone progesterone acts through a 'non-genomic' pathway (Strunker *et al.,* 2011). The response to bolus doses of μM progesterone is composed of a biphasic signal; an initial Ca^{2+} transient, which peaks within the 30s following dosage, is followed by a 'late' plateau component which persists for longer than twenty minutes (Figure 5.1) (Blackmore *et al.*, 1990; Kirkman-Brown *et al.*, 2000; Strunker *et al.*, 2011).

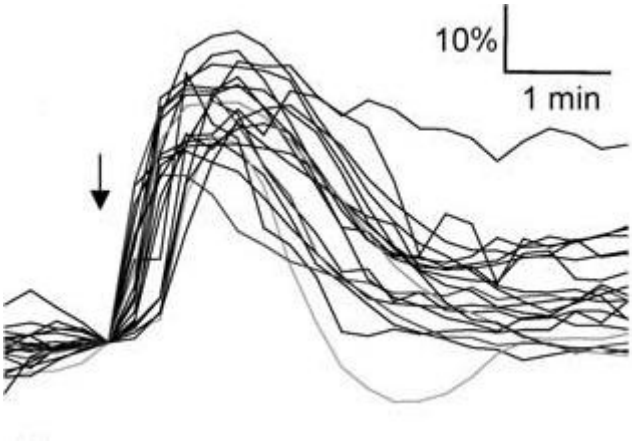

Figure 5.1: Patterns of intracellular Ca^{2+} in response to 3.2μM progesterone. From (Kirkman-Brown *et al.*, 2003). The traces represent the Ca^{2+} signals obtained from 23 donor sperm samples, as measured using fluorimetric measurement of Calcium Green 1-AM. The arrow represents the point at which progesterone was applied.

The transient has been attributed to Ca^{2+} influx via CatSper channels, which have recently been revealed as a direct target of progesterone in human sperm (Lishko *et al.,* 2011; Strunker *et al.,* 2011). However, even when extracellular calcium ions are unavailable, a response to progesterone is still apparent (Bedu-Addo *et al.,* 2007), suggesting that progesterone activates two discrete Ca^{2+} signalling responses in human sperm.

4-Aminopyridine, in its membrane-permeable form, is a highly potent inducer of hyperactivation in human sperm, modulating flagellar bending dramatically, even in

uncapacitated cells. This effect can be observed instantly, and persists until the drug is removed (Gu *et al.*, 2004; Barfield *et al.*, 2005; Bedu-Addo *et al.*, 2008). Particularly active at a concentration of 2mM, single-cell imaging experiments revealed a parallel sustained rise in Ca^{2+} in the vicinity of the sperm neck/midpiece (Figure 5.2) .

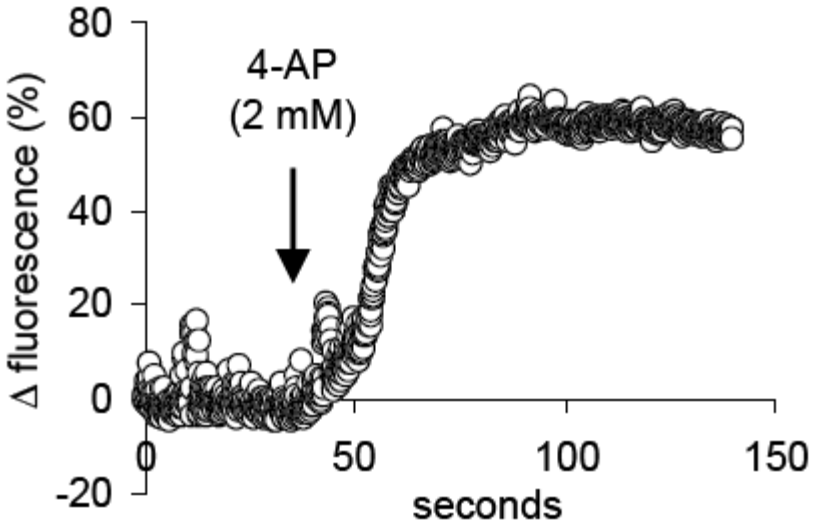

Figure 5.2: Patterns of intracellular Ca2+ in response to 2mM 4-AP. From (Costello, 2010-2011). The traces show the 4-AP-induced increase in fluorescence of Oregon-Green BAPTA-1 (representing the increase in $[Ca^{2+}]_i$).

A well known blocker of voltage-gated K^+ channels (Barfield *et al.,* 2005), the elevation of $[Ca^{2+}]_i$ appears to occur independently of this action, as responses are resistant to 'clamping' of the cell membrane potential with valinomycin (Bedu-Addo *et al.,* 2008). 4-AP is also a weak base, and thereby promotes intracellular alkalinisation. As CatSper current is alkaline dependent (Navarro *et al.,* 2007), it could be proposed that this action of 4-AP was responsible for the ensuing rise in Ca^{2+}. However, the addition of 25mM NHCl, to raise intracellular pH to the same degree as 4-AP, did not preclude a further Ca^{2+} response to 4-AP, suggesting that the potentiation of CatSper was not the main action of the drug (Costello, 2010-2011).

It appears that 4-AP, like progesterone, mobilises stored Ca^{2+} by some mechanism which is yet to be fully elucidated. When calcium ions were omitted from the medium in which the sperm were suspended, i.e. the extracellular concentration was very low at □5µM, a Ca^{2+} response was still observed in 75% of cells. Whilst prolonged incubation with EGTA abolished the response altogether, when 4-AP was applied after only a few minutes then a small, transient response could still be noted in 25% of cells (Bedu-Addo *et al.,* 2008). 4-AP does not induce the acrosome reaction in human sperm cells, which reinforces the concept that sperm regulates Ca^{2+} mediated cellular activities using discrete, hard wired responses (Bedu-Addo *et al.*, 2008).

Whilst store mobilisation alone, by either progesterone or 4-AP, may not provide enough Ca^{2+} ions to significantly impact on any Ca^{2+}-mediated flagellar response, it has been proposed that capacitative calcium entry or store-operated calcium entry (CCE and SOCE respectively) may be responsible for translating store mobilisation into a significant rise in calcium ion concentration around the base of the flagellum (discussed in Chapter 8).

Activators of adenylate cyclase, such as 2'-deoxyadenosine, have a stimulatory effect on sperm motility (Moohan *et al.,* 1995). Phosphodiesterase inhibitors, such as pentoxyfilline and IBMX, restrict the breakdown of intracellular cAMP, thereby exerting their effect downstream on the same pathway, resulting in a similar positive effect on sperm movement (Jiang *et al.*, 1984; Tesarik *et al.*, 1992; Tournaye *et al.*, 1994; Calogero *et al.*, 1998; Fisch *et al.*, 1998; Nassar *et al.*, 1998).

It is unclear to what degree the downstream effects on motility produced by cAMP and Ca^{2+} are shared. One may activate the other, for example cAMP will open nucleotide-gated Ca^{2+}

channels (De Jonge and Barratt, 2006), and the target of calcium ions, that is calmodulin, can also influence enzymes that result in the production of cAMP (Oehninger and Kruger, 2007). However, both in humans and other mammals, their actions on the axoneme and motility have been shown to be distinct. Experiments in bull sperm revealed that, whilst essential for the induction of capacitation and the activation of motility, the AC/cAMP/PKA signalling pathway did not influence hyperactivation specifically. Only those agents affecting intracellular calcium ion levels directly contributed to hyperactivation. Furthermore, their effects were not due to any tyrosine phosphorylation, suggesting that calmodulin must have other flagellar targets (Marquez and Suarez, 2004; Marquez and Suarez, 2008). In humans, a study in which sperm were capacitated and hyperactivated by suspending them in a commercial IVF medium, showed that neither of these effects were controlled by PKA, although tyrosine phosphorylation did increase with these events in this case (Moseley *et al.,* 2005). However, these results are contradictory to those of other studies which have documented a specific influence on the kinematic parameters which define hyperactivation (that is VCL, ALH and LIN) by phosphodiesterase inhibitors (Kay *et al.,* 1993).

In order to be feasible for routine clinical application, a hyperactivation assay would have to be able to elicit maximal hyperactivation very quickly. In order to establish whether or not this could be accomplished using the agonists discussed above, that is, progetsreone, IBMX and 4-AP, each was monitored for its efficacy. Influences on motility were studied with respect to both the degree to which they modulated VCL, ALH and LIN and hyperactivation, and the proportion of cells in which they were effective.

5.2 Experimental procedures

Sperm samples for this chapter were sourced from research donors whose ejaculates were considered 'normal' according to WHO criteria (WHO, 1999). Sperm preparation methods and the assessment of hyperactivation using CASA are detailed in Chapter 4. Cells were resuspended in CM for testing, the recipe for which is also presented Chapter 4. Briefly, prepared samples were divided into four aliquots: control, progesterone, IBMX and 4-AP. Each reagent was applied to samples only immediately prior to CASA assessment, and aspirated four or five times before loading onto the microscope slide to ensure homogenous distribution of both the drug and the cells. Final concentrations were: 3.6µM for progesterone, 100µM for IBMX and 2mM for 4-AP.

5.3 Statistics

As all data obtained for VCL, ALH, LIN and % hyperactivation were found to be approximately normally distributed (using the Kolmorogov-Smirnoff test) and of approximately homogenous variance (*F*-test), differences between samples were analysed using the Paired sample *t*-test. Mean percentage increase in hyperactivation was a parameter which showed significant heterogeneity of variance amongst the progesterone, IBMX and 4-AP treated groups, therefore the Wilcoxon signed-ranks test was used to examine differences in these cases. All statistical tests were carried out in SPSS version 17.

5.4 Results

The effects of 3.6µM progesterone, 100µM IBMX and 2mM 4-AP on the individual motion parameters that comprise the algorithm for hyperactivation, that is VCL, ALH and LIN, and

hyperactivation itself, are detailed in Table 5.1. All three reagents significantly increased VCL and ALH. Progesterone and 4-AP, but not IBMX, significantly reduced LIN.

IBMX exerted the largest effect on VCL. This was significantly different to that of the progesterone induced response (*P*=0.000), but not different to that of 4-AP (*P*=0.476). 4-AP influenced ALH to the greatest extent, and the mean ALH in the presence of 4-AP was significantly higher than the ALH in both the progesterone and IBMX treated samples (*P*= 0.022 and *P*= 0.002 respectively). Similarly, the mean LIN of the 4-AP treated samples was significantly lower than it was when either progesterone or IBMX was applied (*P*= 0.003 and *P*= 0 respectively).

Hyperactivation was significantly increased by IBMX and 4-AP, but not by progesterone. The mean percentage hyperactivation was highest in the samples treated with 4-AP, and although this value was significantly different to the progesterone treated group (*P*=0.035), it was not significantly different to that induced by IBMX (*P*=0.142). Similarly, the mean percentage *increase* in hyperactivation was not significantly different between the 4-AP and IBMX groups (Figure 5.4).

76% of samples responded, in terms of hyperactivation, to IBMX and 4-AP, whilst progesterone, the weakest stimulant of hyperactivation under these conditions, only promoted an increase in hyperactivation in 59% of samples (Table 5.2). On only one occasion did a sample respond to IBMX but not 4-AP, and vice versa. Two samples did not exhibit an increase in hyperactivation levels following the application of any of the three agents.

	VCL (µms^{-1})	ALH (µm)	LIN (VSL/VCL) x 100	Hyperactivation (%)
Control	120.1 ± 5.5	4.7 ± 0.3	59.7 ± 2.1	10.1 ± 2.3
Progesterone	127.1 ± 5 ^{s}	5.3 ± 0.2 ^{s}	56 ± 2.1 ^{s}	12.4 ± 2.4
IBMX	138.8 ± 5.1 ^{s}	5.2 ± 0.2 ^{s}	58 ± 1.9	16 ± 2.7 ^{s}
4-AP	136 ± 7.6 ^{s}	5.8 ± 0.3 ^{s}	47.7 ± 2 ^{s}	19.3 ± 3.7 ^{s}

Table 5.1: Influences of hyperactivation inducers on individual kinematic parameters. Values are mean plus or minus SEM for 17 donor ejaculates. Entries marked with ^{s} indicate a significant difference from the control. In terms of hyperactivation specifically, the IBMX and 4-AP treated groups were significantly different to the control (*P*=0.003 and *P*=0.002 respectively), whilst progesterone did not induce a significant difference in hyperactivation compared to the control (*P*=0.125).

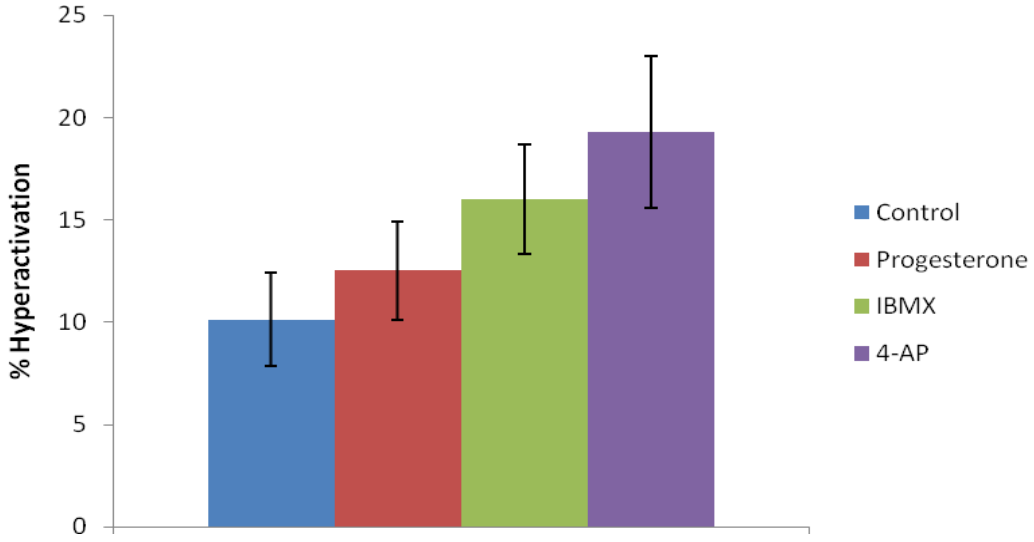

Figure 5.3: Hyperactivation levels in response to progesterone, IBMX and 4-AP. *n*= 17 and error bars represent mean ±SEM.

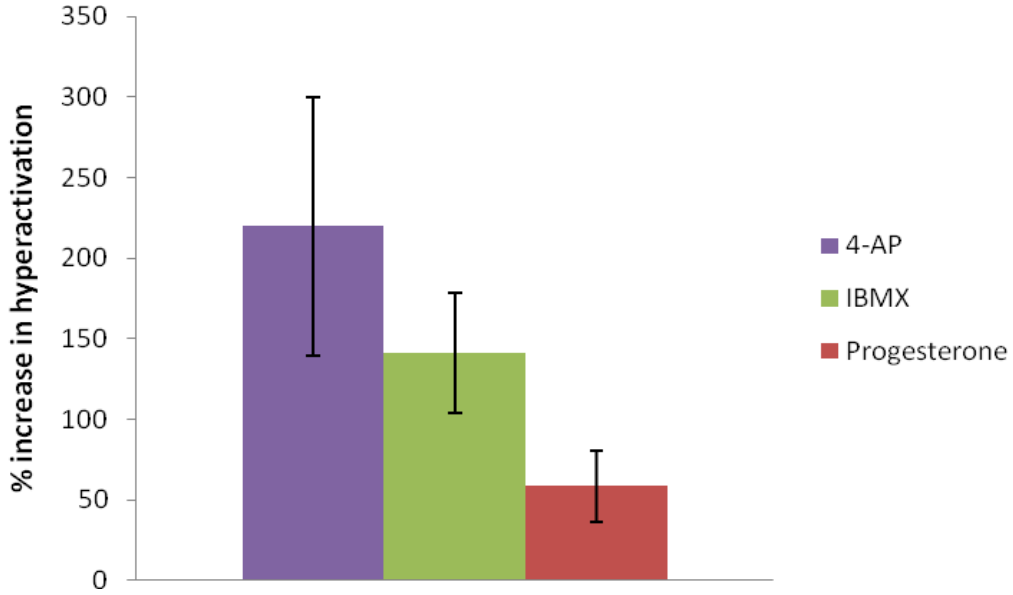

Figure 5.4: Percentage increase in hyperactivation following treatment with progesterone, IBMX and 4-AP. Values represent the *mean* percentage increase in from the control that each stimulant exerted on hyperactivation. *n*= 17 and error bars represent mean ±SEM. The progesterone group is significantly different to the 4-AP and IBMX groups (*P*=0.004 and *P*=0.19), but they are not significantly different from one another (*P*=0.301).

Sample	Control HA (%)	Response to Progesterone	Response to IBMX	Response to 4-AP
1	23.5	✓	✓	✗
2	1.5	✗	✓	✓
3	7.5	✓	✓	✓
4	6	✓	✓	✓
5	1	✓	✓	✓
6	3	✓	✓	✓
7	29	✗	✗	✗
8	10	✗	✓	✓
9	9	✓	✓	✓
10	5.5	✓	✗	✗
11	3	✓	✓	✓
12	6.5	✗	✗	✗
13	1	✓	✓	✓
14	24	✗	✗	✓
15	10.5	✓	✓	✓
16	6	✗	✓	✓
17	30	✗	✓	✓

Table 5.2: Hyperactivation responses by sample to progesterone, IBMX and 4-AP. The 10th centiles for the average percentage increase were calculated using only values where the treated hyperactivation level was higher than that of the control (i.e. not including any negative values). The thresholds were calculated as 9%, for progesterone, 9% for IBXM and 23% for 4-AP, and anything above these thresholds was considered to be a 'response.' Ticks show responses, crosses depict the absence of a response.

5.5 Discussion

Progesterone, in comparison to 4-AP and IBMX, was the weakest stimulator of hyperactivation under these experimental conditions. Whilst the steroid significantly altered each individual motion parameter, that is, VCL, ALH and LIN, this did not translate into a significant difference in hyperactivation (Table 5.1). It is possible, however, that a larger sample size may have shown this modest increment to be significant.

It could be considered surprising, given the well documented influence of progesterone on intracellular calcium ion levels (Blackmore *et al.*, 1990; Baldi *et al.*, 1991; Kirkman-Brown *et*

al., 2003) that the effects of the steroid on hyperactivation under these experimental conditions were not more substantial. Previous investigators have documented an ability of progesterone to induce hyperactivated motility in human sperm (Uhler *et al.*, 1992; Jaiswal *et al.*, 1999), whilst others could not confirm such an action (Kay *et al.*, 1994; Wang *et al.*, 2001).

A possible explanation as to why progesterone did not promote a significant increase in hyperactivation is that the large calcium transient, which typically peaks within one minute following the application of a bolus of progesterone at micromolar doses (Kirkman-Brown *et al.*, 2000), was 'missed' when screening for hyperactivation using the method applied here. For example, in the time it took to load a microscope slide, focus the microscope, allow any 'drift' to stop, and scan a sufficient number of cells, the initial Ca^{2+} transient (and corresponding burst of hyperactivation) may have been over. This would mean that only the moderate, plateau component of the Ca^{2+} signal was captured by CASA.

Progesterone when presented as a bolus seems to impose on cells some kind of refractory period. In vivo, the sperm will not encounter progesterone in this manner; instead, secretion of the steroid by the cumulus oophorus should result in the formation of a concentration gradient along the length of the oviduct (perhaps even extending into the uterus), meaning that sperm in the female tract should encounter progesterone at very low doses (nM) initially, followed by progressively larger ones upon approach to the oocyte. The reproduction of such a setup in vitro has shown that an altogether different pattern of Ca^{2+} signals and hyperactivation are produced under these conditions; a slowly developing rise in Ca^{2+}, as opposed to a high-amplitude transient, is accompanied by Ca^{2+} oscillations in a proportion of cells (Harper *et al.,* 2004). Therefore, the expression of hyperactivation may

vary depending on how sperm meet progesterone, and the effects of progesterone on hyperactivation in the female are likely to be entirely different to the scenario in vitro.

Moreover, in vivo, sperm are exposed to progesterone in parallel with other stimulatory components of the reproductive tract and follicular fluid. For example, in one study, follicular fluid induced an increase in hyperactivation, whereas progesterone did not (Wang *et al.*, 2001). Nitric oxide in particular has been shown to act synergistically with progesterone to enhance and prolong Ca^{2+} signals and corresponding flagellar bending (Machado-Oliveira *et al.*, 2008; Lefievre *et al.*, 2009). Therefore, it may be that, in practice, the complex effects of progesterone on motility involve too many subtleties to permit the generation of a rapid and accurate assay using CASA.

4-AP and IBMX, in contrast, evoked a significant and sustained hyperactivation response (Table 5.1). Whilst these agents have no physiological relevance *per se*, they may offer a means by which to interrogate the signalling pathways involved in the control of hyperactivation. Both 100µM IBMX and 2mM 4-AP induced a rise in hyperactivation in over 75% of samples treated (Table 5.2). With regards to IBMX, two of the four samples which did not respond to treatment with the drug exhibited already high levels of hyperactivation in the control (over 20%), and the same applied in the case of 4-AP.

Whilst both agents therefore appear to be effective activators of hyperactivation, the specific kinematics in the 4-AP and IBMX treated samples were not equivalent. For example, whilst both drugs significantly raised VCL, only 4-AP acted to significantly reduce LIN. In practice, this means that the sperm exposed to IBMX displayed a greater degree of space gain and forward movement than those treated with 4-AP. The increased flagellar

bending induced by 4-AP (reflected in the high ALH) diminished forward progression; a feature that would be particularly prominent when beating was also asymmetrical.

The IBMX group showed a significantly higher BCF than the control group (28.2Hz vs. 26.8Hz respectively, *P*= 0.02), indicating that much of the velocity increase here was mediated by faster tail beating. In contrast, 4-AP treated sperm samples increased their hyperactivation at the *expense* of beat frequency, which was significantly lower than in the control (24.7 vs 26.8, *P*= 0.001). The increase in velocity in these cells must therefore have arisen as a direct result of the force generated by deeper flagellar excursion. Since it is this action specifically which is thought to impart the mechanical capabilities necessary for passage through viscoelastic substances and the cumulus and zona, and not merely faster swimming, it would appear that 4-AP is a more effective inducer of 'genuine' hyperactivation than is IBMX. Whilst very hyperactivated sperm suspended in low viscosity medium produce trajectories which look highly erratic, in a viscous or viscoelastic substance these may translate into effective swimming, burrowing and 'drilling', actions, as may they facilitate direction changes as part of the chemotatic process.

4-AP also produced the highest mean highest and mean percentage increase in hyperactivation (Table 5.1, Figure 5.4), rendering the agonist most capable of inducing this flagellar phenomenon. Thought to act via its store mobilising ability, 4-AP may also be a tool with which to identify men, for the purpose of further analysis, who have problems in generating store-mediated Ca^{2+} signals specifically. Indeed, such a pathology has been alluded to in a previous study involving asthenozoospermic patients (Espino *et al.*, 2009).

Chapter 6: Population-specific expression of hyperactivation

6.1 Introduction

Hypeactivation is a parameter which is not routinely measured during basic andrology diagnostics, and therefore little has been established regarding the incidence of hyperactivation disorders or their prevalence amongst different groups of men. For example, it is unclear how closely the ability to induce hyperactivated motility correlates with the parameters measured in a semen analysis. It may be, for example, that men whose semen characteristics are suboptimal (perhaps, in terms of motility, due to an artefact of the semen as opposed to any pathology of the sperm) can readily induce hyperactivation, whilst other men who exhibit normal or nearly normal semen characteristics (including motility) have difficulty eliciting the more forceful flagellar movement that is associated with hyperactivation. By performing only a semen analysis, it is possible that an important step in determining a man's fertility may be omitted.

Three distinct populations were available in which to probe for any differences in hyperactivation between men grouped according to semen analysis and fertility: research donors, whose semen parameters were normal yet little was known about their fertility, IVF patients, whose semen parameters were normal or approaching normal limits yet who formed part of an infertile couple, and ICSI patients, whose semen parameters were identified as abnormal or whose sperm had failed to fertilise oocytes during a previous cycle of conventional IVF. 4-AP, verified as the most potent inducer of hyperactivation in human sperm (Chapter 5), was used as an inducer to elicit maximal levels for the purposes of screening.

6.2 Experimental procedures

Sperm samples for this chapter were sourced both from research donors and male patients undergoing IVF or ICSI treatment. Methods for selection of donors and allocation of patients to standard IVF or ICSI are detailed in Chapter 4, as are sperm preparation methods and the assessment of hyperactivation using CASA. All donor sperm samples were prepared using clinical media: Cook Sperm Medium™ for those being directly compared with IVF samples and Cook Gamete Buffer™ for those being directly compared with ICSI samples. The purpose of this was to ensure that the conditions under which patient samples were measured were exactly replicated. 4-AP was added immediately prior to CASA assessment, at a final concentration of 2mM, and the sample aspirated four or five times to ensure homogenous distribution of both the drug and the sperm cells before loading onto a microscope slide. All surplus IVF samples donated to research were screened for hyperactivation, however, approximately one third of the ICSI samples donated were not assessed. Such samples were of very poor quality and it was therefore not possible to record 800 motile cells as per the protocol outlined in Chapter 4.

6.3 Statistics

The Kolmorogov Smirnoff test was used to test for normal distributions. Upon finding that two data sets had distributions which were significantly different from a normal distribution, for consistency, a non-parametric was used to test for statistically significant differences between two groups in all cases. The Mann-Whitney *U* test was selected because all comparisons were between unpaired data, that is, as opposed to between repeated measures of the same individuals. All statistical tests were carried out in SPSS version 17.

6.4 Results

Table 6.1 summarises the hyperactivation levels observed in each population both in the presence and absence of 4-AP. The Mann-Whitney *U* test returned the result that there was no evidence that the hyperactivation levels of the IVF patient group came from a different distribution to those of the research donor cohort, i.e. they were not statistically different (Figures 6.1 and 6.2). This was the case both when the samples were untreated, and when they were tested in the presence of 4-AP.

Conversely, the ICSI patients exhibited significantly lower levels of hyperactivation than their research donor counterparts; a difference which was statistically significant in the presence of 4-AP-treated and approaching levels of significance without it (Figures 6.3 and 6.4).

The hyperactivation levels in the IVF patient samples were significantly higher than they were in the ICSI population. However, this is not a valid comparison as Cook Sperm Medium™ specifically promotes hyperactivation and capacitation, whilst Cook Gamete™ Buffer is designed to repress these processes.

With respect to the way in which each population responded to 4-AP, individual values can be found in Table 6.2. There were no significant differences between the mean percentage response to 4-AP in the IVF patients and the research donors in Cook Sperm Medium™ (P=0.77), nor was there any difference between the ICSI patients and the research donors in Cook Gamete Buffer™ (P=0.869). In addition, the proportion of ICSI samples which responded to 4-AP was similar to the proportion of donor samples which responded (79%

vs. 82%). There were no significant differences between the percentage response of samples in Gamete Buffer™ and those in Sperm Medium™.

Population	No. of samples	Mean % hyperactivation	Std deviation
IVF patient samples untreated	86	8.9	7.6
IVF patient samples 4-AP	86	18.7	13.3
Donor samples in Sperm Medium untreated	30	8.6	7.2
Donor samples in Sperm Medium 4-AP	30	15.1	9.4
ICSI patient samples untreated	24	5.1	6.7
ICSI patient samples 4-AP	24	10.1	11.7
Donor samples in Gamete Buffer untreated	22	6.9	6.9
Donor samples in Gamete Buffer 4-AP	22	13.9	10.1

Table 6.1: Hyperactivation levels in research donors and ART patients. IVF patient samples arrived from the Assisted Conception Unit pre-prepared in Cook Sperm Medium™. Therefore, in order to ensure that a direct comparison was being made with the research donor samples, these were also prepared in this commercial medium. Similarly, any research samples being directly compared with ICSI samples were prepared in Cook Gamete Buffer ™.

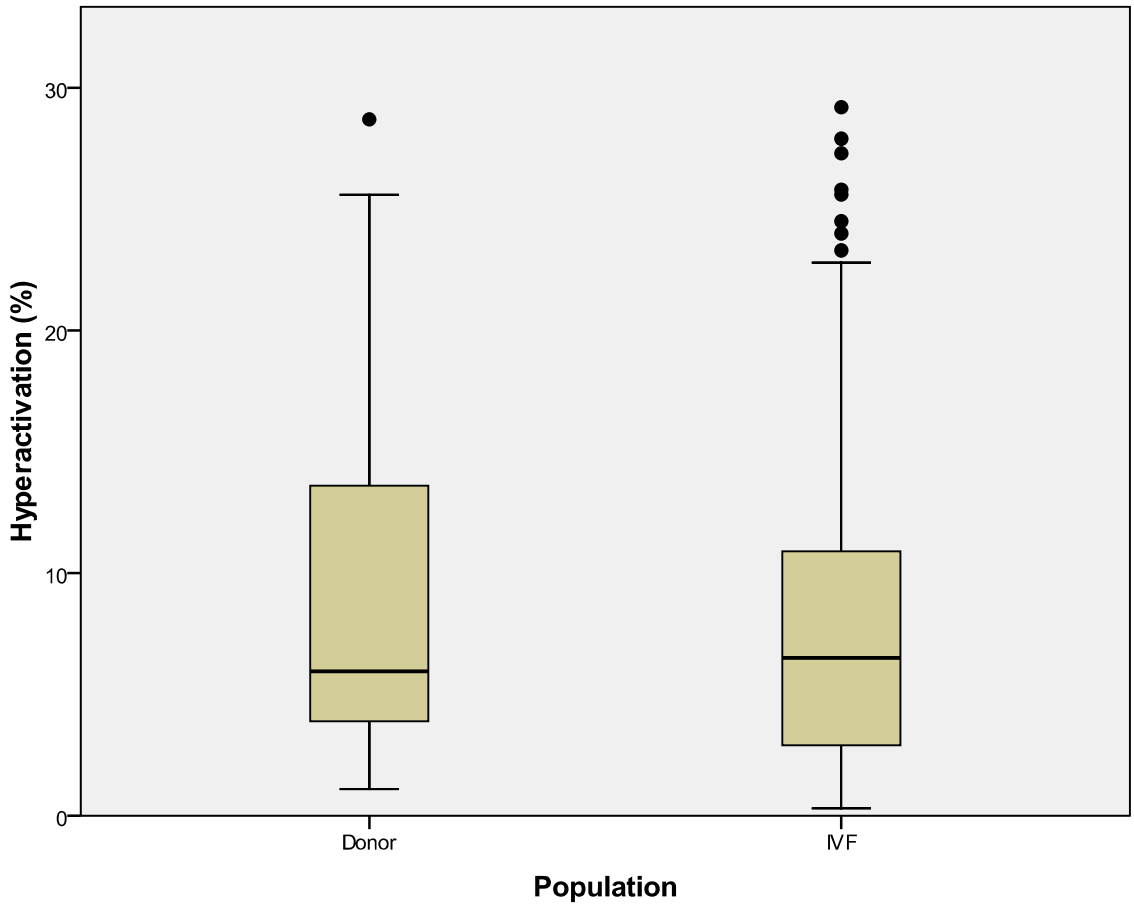

Figure 6.1: Hyperactivation in untreated research donor vs. IVF patient sperm samples. All measurements were recorded in Cook Sperm Medium™. The interquartile range is represented by the box, whilst the line through the box is the median. The whiskers correspond with the largest or smallest values within 1.5 interquartile ranges of the edges of the box, and the outliers are depicted as dots. The two groups are not significantly different (*P*=0.970).

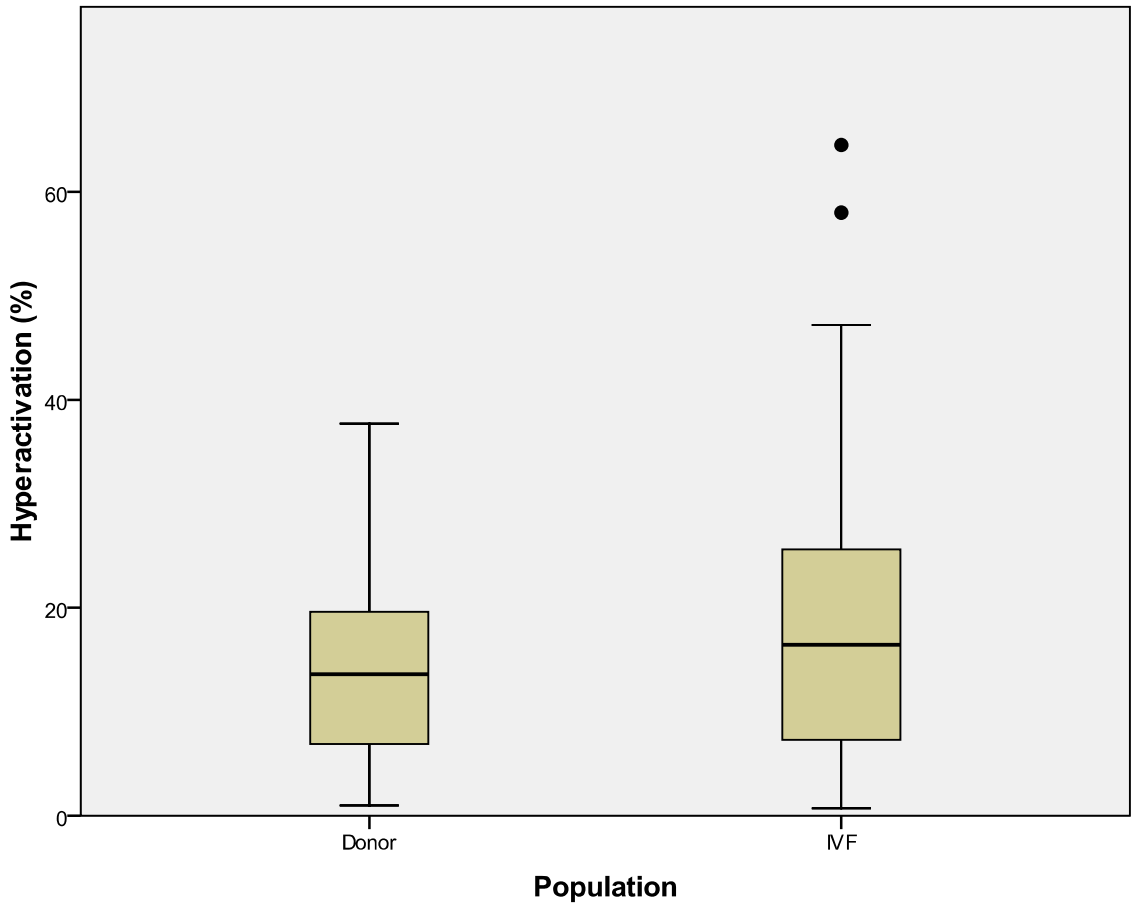

Figure 6.2: Hyperactivation in 4-AP-treated research donor vs.IVF patient sperm samples. All measurements were recorded in Cook Sperm Medium™. The interquartile range is represented by the box, whilst the line through the box is the median. The whiskers correspond with the largest or smallest values within 1.5 interquartile ranges of the edges of the box, and the outliers are depicted as dots. The two groups are not significantly different (*P*=0.304).

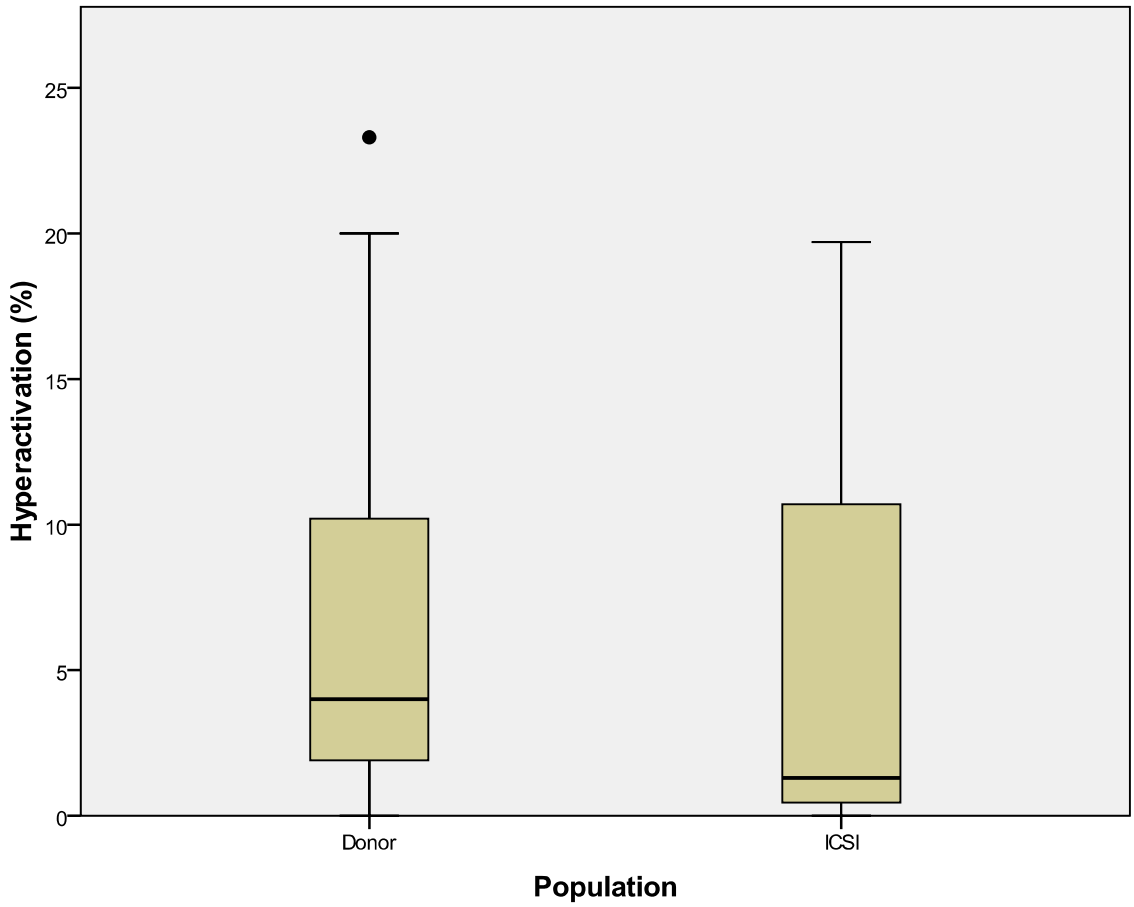

Figure 6.3: Hyperactivation in untreated research donor vs. ICSI patient sperm samples. All measurements were recorded in Cook Gamete Buffer™. The interquartile range is represented by the box, whilst the line through the box is the median. The whiskers correspond with the largest or smallest values within 1.5 interquartile ranges of the edges of the box, and the outliers are depicted as dots. The two groups are not significantly different (*P*=0.073).

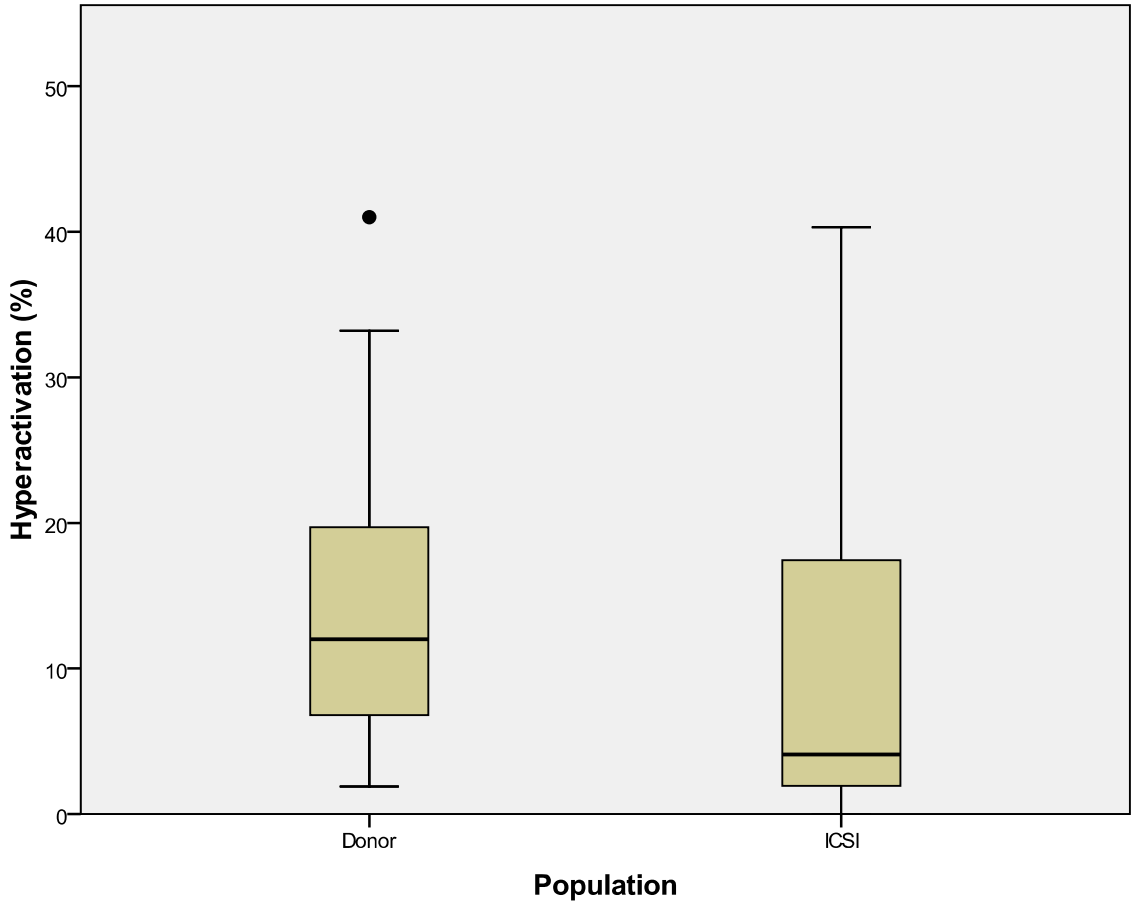

Figure 6.4: Hyperactivation in 4-AP treated research donor vs. ICSI patient sperm samples. All measurements were recorded in Cook Gamete Buffer™. The interquartile range is represented by the box, whilst the line through the box is the median. The whiskers correspond with the largest or smallest values within 1.5 interquartile ranges of the edges of the box, and the outliers are depicted as dots. The two groups are statistically significantly different (*P*=0.048).

	Mean % response to 4-AP	Proportion of samples showing 'response'
IVF patients	211 ± 33	88%
Donors in Cook Sperm Medium	151 ± 39	77%
ICSI patients	189 ± 46	79%
Donors in Cook Gamete Buffer	409 ± 212*	82%

Table 6.2 Responses to 4-AP in patient and donor sperm. Shown are the mean responses (±SEM) of each population to 4-AP, that is, the average percentage increase in hypeactivation following the application of 4-AP. Taking an arbitrary value of a 20% increase in hyperactivation as a response, the proportion of samples in each group which responded to 4-AP is also shown. * Very high SEM means that outlying very high value(s) are influencing the overall mean, as the ICSI patients and donors in Gamete Buffer™ are not significantly different (*P*=0.869).

6.5 Discussion

Following treatment with the stimulant 4-AP, the hyperactivation levels displayed by the ICSI cohort were significantly lower than those of the research donors, suggesting that sperm samples which have been assigned to ICSI exhibit lower levels of hyperactivation than samples in which no semen abnormalities have been observed. Similar evidence, namely that hyperactivation-specific defects are more prevalent in oligo-, astheno- and teratozoospermic patients, has been documented previously (Burkman, 1984; Pilikian *et al.*, 1991). It is possible that the reduction in hyperactivation in the ICSI patients was due to deficiencies in flagellar architecture, especially, for example, if asthenozoospermia in semen was the indication for ICSI in the first place. This could also be attributed to inefficient operation of the signalling pathway underlying hyperactivation, however, the fact that it was only the absolute values for hyperactivation which were lower, and not the ability of these samples to *respond* to 4-AP suggests that this is less likely. This ability to respond was similar to the donor population both in terms of the mean percentage response and the number of samples in which a response could be observed, suggesting that the signalling pathway exploited by 4-AP at least, is not specifically deficient.

There was no significant difference between hyperactivation in the research donors and the IVF patients, even in the presence of 4-AP. Whilst the specific semen analysis results for the IVF cohort are not shown, these patients, by virtue of the fact that they were selected for IVF as opposed to ICSI in the first place, had normal or nearly normal semen characteristics (detailed in Chapter 4). They were, therefore, very similar in this respect to the research donor group.

The fact that the IVF patients and the research donors were found to be equivalent in terms of their ability to induce hyperactivation indicates that the IVF patients are no different to 'normal' men (with unknown fertility status but who are not being investigated/treated for infertility) in this respect. What this means is that there was no evidence of any *general* hyperactivation pathology in the IVF patients which may have been responsible for/contributory to their infertility. Certainly the majority of these patients will have already had confirmed female factors to specifically account for their infertility.

In conclusion, what these small sample sizes are suggesting is that the expression of hyperactivation in a sample is, in a very broad sense, linked to semen characteristics in that normal semen samples are more likely to yield sperm which have a good capacity for hyperactivation, whereas sperm sourced from abnormal semen samples will be less likely to exhibit pronounced hyperactivation. Whilst low levels of hyperactivation are prevalent in the ICSI population, any detriment in this respect may be secondary to a far more serious sperm defect(s). There has purposely been no attempt made to establish threshold levels for 'normal' or 'abnormal' hyperactivation in this chapter, as without corresponding information on fertility, any arbitrary limits set according to, for example, percentile value, are essentially meaningless. Whether or not there are any negative clinical outcomes associated with samples which exhibit the very lowest levels of hyperactivation, is specifically discussed in Chapter 7 which follows.

Chapter 7: The value of a pre-IVF hyperactivation screen

7.1 Introduction

Data gathered in the previous experimental chapter highlighted that the expression of hyperactivated motility was linked with semen analysis: samples which were selected for ICSI due to a poor semen parameters expressed lower levels of hyperactivation than those which were deemed suitable for IVF. What it was unable to do, however, was relate the expression of hyperactivation to fertilising potential, and indeed the focus of this chapter is to examine the prognostic potential of a pre-IVF hyperactivation assay.

Using 4-AP, a potent agonist known to promote maximal levels of hyperactivation instantaneously (demonstrated in Chapter 5), such an assay could be carried out in seconds, on the day of treatment and using the specific ejaculate scheduled for insemination, thereby eliminating any discrepancies arising from intra-ejaculate variation.

Importantly, if the experiments carried out in this chapter were to highlight that a hyperactivation assay has negative predictive power, then it may have the ability to identify samples which, despite a normal or borderline semen analysis, have a poor chance of succeeding at IVF. Embryologists regularly redirect treatment from IVF to ICSI if the quality of the semen has deteriorated since it was initially tested, and a similar conversion could be offered if a sample scored poorly on the hyperactivation test.

Poor or ‘failed’ fertilisation, usually considered less than 25% of oocytes normally fertilised (Mortimer and Mortimer, 2004), afflicts approximately 5% of IVF cycles (10% in the Assisted Conception Unit at Dundee, not taking into account oocyte maturity). Whilst such

events may be interesting from a diagnostic perspective, an entire treatment cycle has effectively been wasted. If a hyperactivation screen could help prevent even a small number of failed fertilitsations, then it may be worth employing such extended sperm testing not only in specialist or research andrology laboratories, but in the IVF laboratory as well.

7.2 Experimental procedures

7.2.1 Patient selection, ovarian stimulation and oocyte recovery

Patient indications for subfertility/infertility included tubal disease, polycystic ovarian syndrome, endometriosis, mild male factor and unexplained. Female patient age ranged from 24 to 42, with the mean age being 34.4. Ovarian stimulation was undertaken using either the GnRH agonist or antagonist protocol, with gonadotrophin dosage of 150iu/day up to 300iu/day depending on the results of any previous stimulation, female age, risk of hyperstimulation and BMI. hCG was administered when two follicles reached 18mm or greater, and trans-vaginal egg collection was carried out 36 hours later. Cycles which yielded fewer than four eggs were excluded from the study, and a mean of 11.4 eggs were recovered per cycle. The male patients used in this chapter are the same cohort as the IVF patients in Chapter 6.

7.2.2. Sperm preparation

The methods for sperm procurement, semen analysis, assignment of samples to IVF or ICSI, and sperm preparation are detailed Chapter 4.

7.2.3 Insemination

All eggs, regardless of their maturity status as assessed by the evaluation of the oocyte-corona-complex, were inseminated at 3:45pm on the day of retrieval, that is, between five and seven hours post both egg collection and sperm preparation. Up to 8 oocytes per well were inseminated with approximately 150 000 sperm, in a final volume of 0.7ml of Cook Fertilisation Medium™ under tissue culture oil.

7.2.4 CASA assessment of hyperactivation

A detailed protocol for the measurement of hyperactivation can be found in Chapter 4. Samples were screened both for spontaneous levels of hyperactivation, and their expression of hyperactivation under the influence of 4-AP. 4-AP was chosen as an agonist with which to elicit the maximum possible levels of hyperactivation based on the results detailed in Chapter 5, which indicate its high potency in human sperm. At a final concentration of 2mM, 4-AP was applied to samples only immediately prior to CASA assessment, and aspirated four or five times before loading onto the microscope slide to ensure homogenous distribution of both the drug and the sperm cells.

7.2.5 Determination of fertilisation rate

Assigning an appropriate value for fertilisation in the context of this assay was complex; a fact which has either been overlooked or inadequately explained by many previous investigators. As a marker of quality control in the embryology laboratory, the fertilisation rate is conventionally considered to be the number of normally fertilised eggs obtained, that is, zygotes displaying two pronuclei, divided by the number total number of eggs inseminated (multiplied by one hundred) (Mortimer and Mortimer, 2004). However, since

it is only sperm that are being assessed here, it does not seem appropriate to use that same definition as an end point.

For example, significant variation in quality between not only each cohort of eggs, but each individual egg means that using fertilisation rate as a 'measure' of sperm capability is essentially uncontrolled. Adjusting for confounding egg factors can alleviate this problem to some extent.

Eggs which are immature or very post mature may fail to show signs of fertilisation, even if sperm have interacted with the oolemma (Gardner *et al.*, 2004; Elder and Dale, 2011). Such eggs should not be included in the total available for fertilisation, as it is impossible to tell whether or not the cumulus and zona have been penetrated by sperm. There is also evidence to suggest that eggs which are grossly morphologically abnormal, for example those containing large vacuoles, are less likely to display signs of fertilisation (Veeck, 1986; Gardner *et al.*, 2004; Elder and Dale, 2011).

Using the visualisation of two pronuclei as the only definition of fertilisation seems similarly inappropriate when the focus of the assay is sperm competency. For example, polypronuclear eggs are not caused by any fault in the sperm, rather the opposite, and even eggs displaying a single pronucleus may have been activated by sperm.

Despite the existence of numerous 'grey areas' the following tables were created in order to provide a methodology with which to classify each oocyte inseminated. Whilst this is admittedly an imperfect system, it was nonetheless deemed the best way to assign a fertilisation rate to each cycle with respect to putative sperm performance only.

Egg status 16 hours post-insemination	Explanation of classification
Two pronuclei present	Normal fertilisation has taken place
Three or more pronuclei present	Abnormally fertilised but likely to have been caused by polyspermy or non-extrusion of the second polar body
One pronucleus present	Whilst both parental pronculei should appear simultaneously in a normal zygote, evidence suggests that the majority of 1PN's (up to 80%) have actually been penetrated by sperm.
Two polar bodies visible	Whilst it is possible that a polar body may have fragmented, the presence of two polar bodies, indicating that Meiosis II has been completed, is the second most important indicator of fertilisation after the visualisation of pronuclei

Table 7.1: Eggs classified as fertilised. Constructed using data gathered at 'fert check' sixteen hours post insemination. Any egg conforming to one of these descriptions was considered fertilised. Information sourced from (Levron *et al.*, 1995; Veeck, 2003; Gardner *et al.*, 2004; Elder and Dale, 2011).

Egg status 16 hours post-insemination	Considered receptive to being fertilised?	Explanation of classification
Germinal vesicle	No	Very immature (not yet reinitiated Meiosis I)
No polar bodies visible	No	Immature (still in Meiosis I)
One polar body visible	Yes	Although Meiosis I could have been completed at a late stage of the insemination, this cannot be established. Therefore these eggs are considered to have been receptive to fertilisation.
Empty zona, degenerate or atretic egg	No	Absent or dying egg
Cytoplasmically dark or granular	Yes	May be a sign of a post-mature egg. Prone to polyspermy so still receptive to sperm.
Macro, vacuolated, irregular, fragmented polar body	Yes	Importance of egg morphology for successful fertilisation is debated, however, since in each of these categories there are some examples of fertilised eggs they are included in the total number of available eggs

Table 7.2: Unfertilised eggs. Constructed using data gathered at 'fert check' sixteen hours post-insemination. Unfertilised eggs (that is, eggs which do not meet the criteria outline in Table 6.1) are classified as either 'receptive' to fertilisation or 'non-receptive.' The purpose of making this distinction is to ensure that only those eggs which had a genuine chance of being fertilised are used to calculate the final fertilisation rate. Information sourced from (Levron *et al.*, 1995; Veeck, 2003; Gardner *et al.*, 2004; Elder and Dale, 2011).

The IVF fertilisation rate for a cycle was considered to be:

$$\frac{\text{No. of fertilised eggs (according to Table 7.1)}}{\text{Total number of eggs (that is, all fertilised eggs (according to Table 7.1) plus receptive but unfertilised eggs (according to Table 7.2))}} \times 100$$

7.3 Statistics

Tests of distribution were carried out using the Kolmorogov-Smirnoff test. As a number of variables produced data sets that were significantly different from a normal distribution (Table 7.3), the non-parametric Spearman rank-order correlation test (which gives a value of r_s) was used to test for associations between variables. r_s ranges from -1, though 0, to 1, where -1 is a perfect negative correlation, 0 is no correlation whatsoever, and 1 is a perfect positive correlation. The use of regression analysis, to probe for any 'cause and effect' relationship, was inappropriate here, as neither of the main variables (hyperactivation and fertilisation rate) were specifically set by the experiment. Statistical analysis was carried out using SPSS version 17.

7.4 Results

Upon calculating fertilisation rates using the method outlined in section 7.3, the spread of the data was found to be highly significantly different to that of a normal distribution (Table 7.3 and Figure 7.1). Figure 7.1 shows that data points were concentrated around the top end of the graph, with very low 'outlying' values ('failed' fertilisations) resulting in a mean which was lower than the median.

Variable	No. of observations	Mean	Std Dev	Median	Significantly different from normal distribution?
Fertilisation rate (%)	81	80.1	25.2	88	Yes (*P*=0.01)
Spontaneous hyperactivation (%)	81	8.6	7.7	6	Yes (*P*=0.018)
4-AP-induced hyperactivation (%)	81	18.5	13.5	15	No (*P*=0.293)
% increase in hyperactivation with 4-AP	81	219	315	145.5	Yes (*P*=0)

Table 7.3: Summary of data pertaining to the primary variables explored in the IVF-hyperactivation screen. Data gathered from 81 conventional IVF cycles was used in this study.

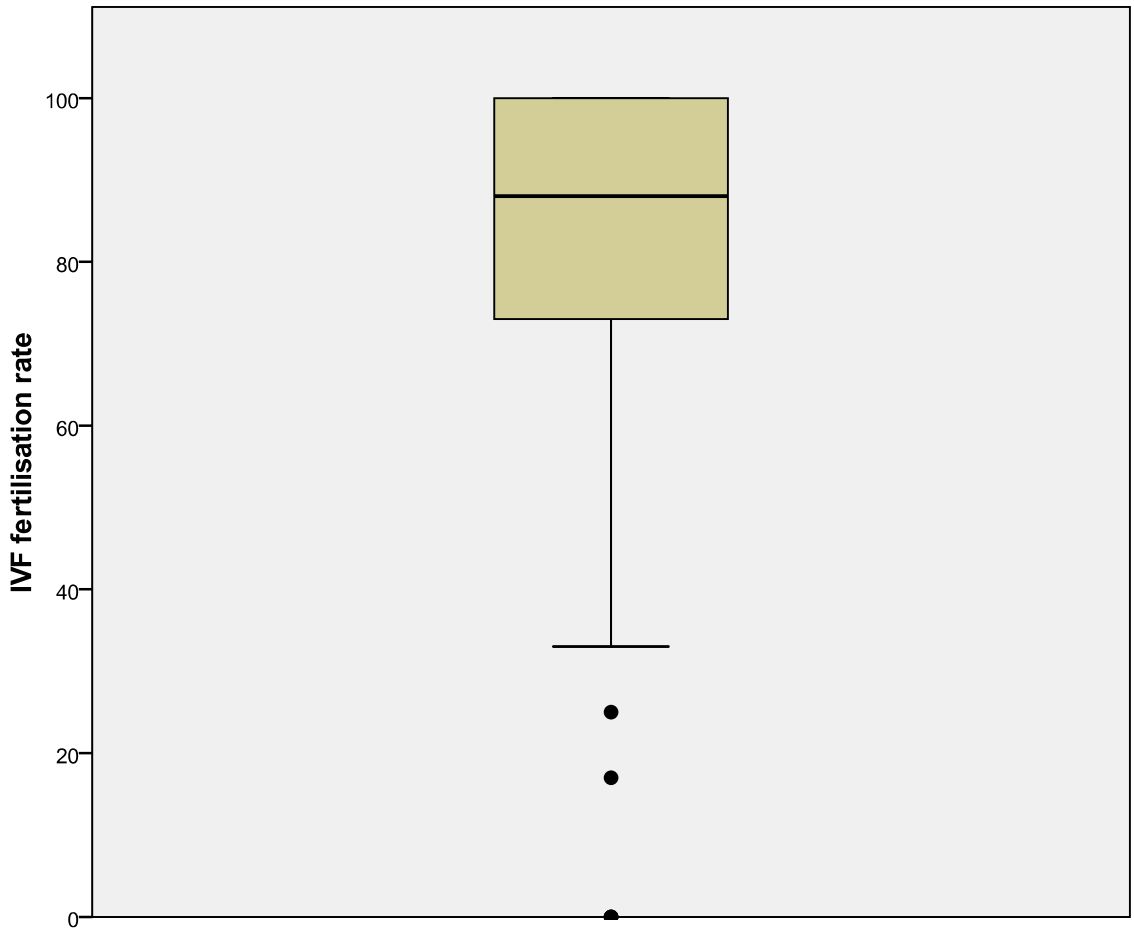

Figure 7.1: IVF fertilisation rates. Boxplot depicts the IVF fertilisation rates calculated using the criteria outlined in Tables 7.1 and 7.2.

The comparison of hyperactivation expression and fertilisation rate achieved at IVF revealed no significant correlation between the two. This was the case for both spontaneous hyperactivation, and that which was induced following exposure to 4-AP. The values for the Spearman rank-order correlations were r_s= 0.05 (*P*= 0.658) and r_s = 0.106 (*P*= 0.345) respectively (Figures 7.2 and 7.3). Similarly, no significant relationship could be noted between the responsiveness of cells to 4-AP, that is, the percentage increase in hyperactivation following application of 4-AP, and fertilisation rate (Figure 7.4).

Considering only the lowest quartiles for hyperactivation levels, both those which arose spontaneously and those which were induced by 4-AP, the mean fertilisation rate was 77% for the spontaneous group (similar to the overall mean fertilisation rate of 80%), and 84% for the 4-AP-treated group (greater than the overall mean). This very clearly indicated the absence of any specific negative associations with hyperactivation and fertilisation rate.

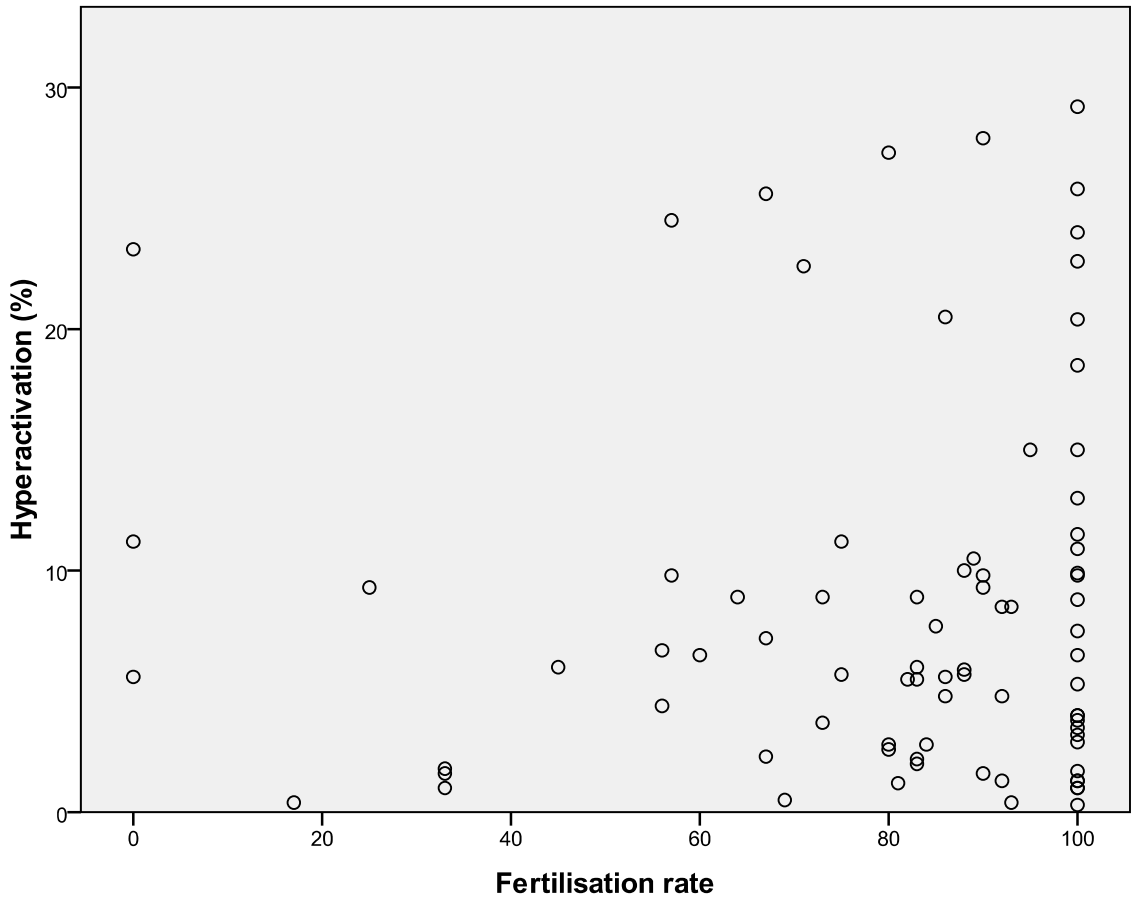

Figure 7.2: Hyperactivation in untreated samples versus fertilisation rate. No significant correlation could be observed between the two; r_s = 0.05, *P*= 0.658.

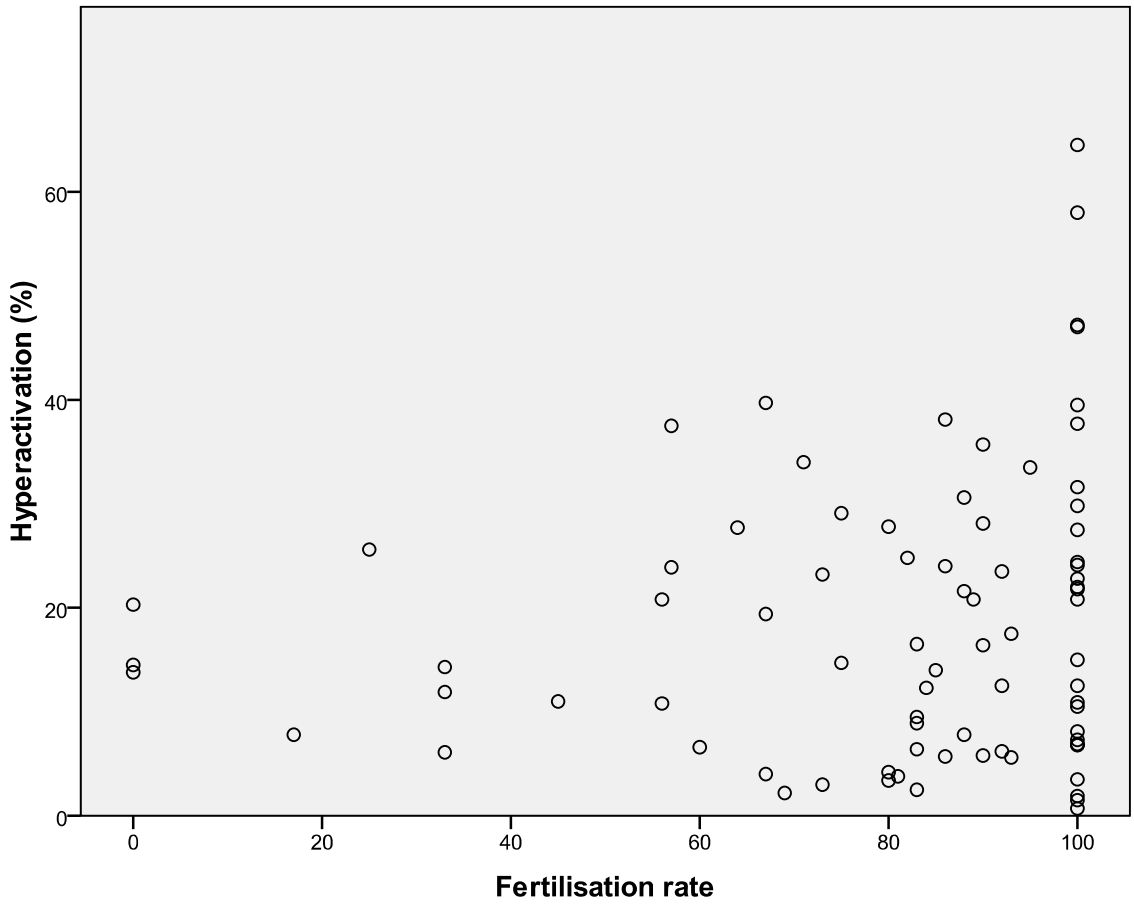

Figure 7.3: Hyperactivation in 4-AP-treated samples versus fertilisation rate. No significant correlation could be observed between the two; r_s= 0.106, *P*= 0.345.

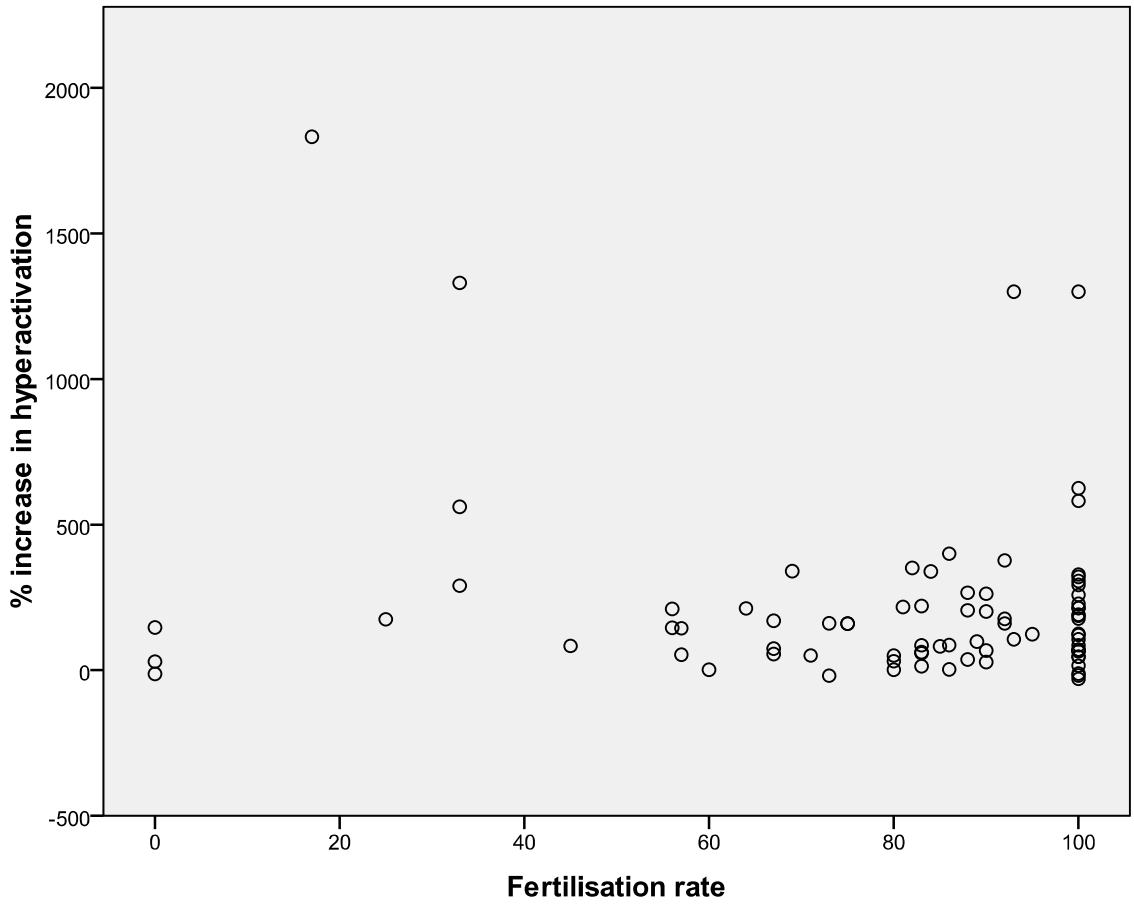

Figure 7.4: Percentage increase in hyperactivation following application of 4-AP. Response to 4-AP was calculated as ((% 4-AP-induced hyperactivation - % basal hyperactivation) x 100 / % basal HA). No significant correlation could be observed between the two; r_s= 0.056 (*P*= 0.617).

Upon finding no meaningful relationship between fertilisation rate and hyperactivation specifically, population-averaged values for individual kinematic parameters were also evaluated for possible associations with fertilisation. Whilst the results are not shown, none of the kinematic measures conventionally used to construct hyperactivation algorithms, that is, VCL, ALH and LIN, were significantly related to fertilisation rate individually. Fertilisation rate was, in fact, unrelated to all of the kinematic measurements recorded, which are outlined in Chapter 4.

7.5 Discussion

None of the experiments carried out in this chapter demonstrated any degree of sensitivity or specificity; that is, 'good' hyperactivators failed to consistently produce high fertilisation rates, nor did low levels of hyperactivation in a sample did not consistently preclude successful fertilisation (Figures 7.2 and 7.3). No significant correlation could be established between fertilisation rate and hyperactivation under these experimental conditions. This applied to the assessment of spontaneous expression of hyperactivation in untreated samples, hyperactivation levels in the presence of 4-AP, and percentage increase in hyperactivation in response to 4-AP.

The fact that the experiments carried out here yielded no indication that sperm samples displaying highest levels of hyperactivation fertilised oocytes in vitro most successfully is perhaps not entirely surprising. For example, it has previously been acknowledged that the highly multi-factorial nature of sperm function dictates that motility parameters alone cannot determine sperm fertilising potential (Tomlinson *et al.*, 1999; Amann and Katz, 2004). Despite established links between hyperactivation and other markers of cell integrity (discussed in Chapter 2), this type of motility can, for example, be readily induced in cells which are deficient in other parameters, like those exhibiting extremely poor morphology. In fact, even globozoospermic samples, despite abnormal midpiece structures, can hyperactivate normally (Aitken *et al.*, 1990).

Moreover, other factors influencing IVF conditions may have also masked the effects of 'good' sperm and confound results in this context. Despite every attempt to exclude oocytes which were obviously immature or defective, possible hidden flaws, such as an abnormal chromosome compliment or any kind of receptor malfunction (Veeck, 2003),

make it difficult to assess sperm performance in a controlled manner. Split cycles (in which half the oocytes undergo conventional IVF and the other half are subjected to ICSI) would allow for better discrimination between sperm and egg factors, but their use is rare (only three times out of 110 cycles in the time period studied). However, even these are imperfect due to the heterogentity in a cohort of eggs.

What could be considered more unusual than the absence of any degree of *sensitivity* (the identification of 'true positives') emerging from the experiments in this chapter, is that even sperm samples with the poorest hyperactivation levels (both in the presence and absence of 4-AP) fertilised eggs successfully. No threshold hyperactivation level, below which fertilisation rates were consistently poor, could be identified. On the contrary, the fertilisation rates acheived by the poorest hyperactivators were in fact equivalent to those of the best hyperactivators.

If not even failure at IVF can be predicted, could it be that the expression of hyperactivation is simply not an essential prerequisite for fertilisation in humans? There is a lack of detailed studies on the motility patterns of sperm recovered from human fallopian tubes, and, understandably, 'in situ' studies are absent. Indeed the majority of research into the specific relevance of this 'step-up' in motility on fertilisation, that is, studies involving sperm recovered from the oviduct and CatSper work, has been conducted in animal models (Katz and Yanagimachi, 1980; Suarez *et al.*, 1983; Ren *et al.*, 2001; Carlson *et al.*, 2003).

Whilst there is little conclusive data pertaining to the specific biological relevance of hyperactivation in humans, given its critical function in mammals and the similarities in the fertilisation process it seems unlikely that hyperactivation in humans could be a redundant

artefact. More probably, it is that the requirement for *pronounced* hyperactivation becomes less important in an IVF scenario, compared to in vivo.

One possible reason for this could be that passage through the fallopian tubes and navigation towards the oocyte are the fundamental purposes of hyperactivation in humans, and *not* the penetration of the oocyte vestments. If this were the case then the simple elimination of the female reproductive tract, as in an IVF scenario, would explain why hyperactivation levels did not seem relevant in vitro. Much of the evidence in other species, however, specifically suggests that even when only the latter most barriers to fertilisation are present, the expression of hyperactivated motility is still an absolute requirement if those barriers are to be breached (Stauss *et al.,* 1995).

A more plausible rationale with which to justify successful IVF in cases where hyperactivation was low is that the sheer numbers of sperm around the oocyte negate the need for a large proportion of cells to be hyperactive. For example, by using insemination numbers of approximately 150 000, at least a thousand times more sperm will be present in the vicinity of the oocytes compared than in vivo (Elder and Dale, 2011). Under such conditions, a hyperactivation level of only 5% corresponds to 7500 sperm. Even supposing only 10% of these are functionally competent in other aspects, that still leaves 750 ‘good’ sperm situated in immediate proximity to the oocytes. In a volume of only 0.7ml, the collision rate will be high. It is therefore not difficult to see how even samples displaying very low proportions of hyperactivated cells can fertilise eggs comfortably, and indeed one of the purposes of IVF, and not just ICSI, is to make it easier for poorer quality sperm to fertilise eggs. The boxplot depicting fertilisation rate (Figure 7.1) is testament to the fact

that, when variables pertaining to oocytes were controlled (as far as was possible), fertilisation rate at IVF is very high.

There were, even in the 4-AP treated samples, a number of hyperactivation readings which were recorded as very close to zero, however fertilisation was still accomplished. If the expression of hyperactivation is essential for penetration of egg vestments, then how did such samples fertilise oocytes? In these experiments, percentage hyperactivation was specifically defined as the proportion of cells which demonstrated movement characteristics comprising VCL ≥ 150µm/s plus ALH ≥ 7µm plus LIN ≤50% at the specific time point assessed. Such an algorithm was specifically chosen because it had been validated using visual and flagellar analysis of cell behaviour and was designed to encompass all aspects of hyperactivated movement described (Mortimer *et al.,* 1998). Whilst for measurement purposes, hyperactivation of course has to be assigned some sort of mathematical definition; such an 'all or nothing' status in practice may not be truly representative of hyperactivation. For example, it may be that a cell with a lower than threshold ALH may be able to compensate by having a greater VCL, and vice versa. Subtly different styles of motion may all actually appear to be hyperactivated, and indeed be able to accomplish the same thing mechanically. Therefore, just because a sample is recorded as having 0% hyperactivation, it does not necessarily mean that it will be unable to effectively perform any of the tasks dependent on this type of motion. This may have been the case despite no specific relationships having been found between hyperactivation and VCL, ALH or LIN either.

It also seems possible that the hyperactivation algorithm used was too stringent. For example, whilst any algorithm based around high VCL, high ALH and low LIN algorithm will

accomplish the task of separating the 'best' hyperactivators from the 'worst' hyperactivators , if the 'worst' perform just as efficiently at IVF as the 'best,' as was the case in this assay, then it seems possible that the criteria was set too high. In practice, the movement characteristics required to penetrate the cumulus and the zona may not accurately match those stipulated by the algorithm applied. Perhaps not even the samples displaying the lowest levels of hyperactivation were actually afflicted by any hyperactivation pathology, thereby explaining why fertilisation proceeded normally. In fact, hyperactivation-specific defects, that is, the inability to exhibit hyperactivated movement whilst activated movement appears normal, may, as noted in Chapter 5, be rare.

In summary, in the context of the IVF program at the Assisted Conception Unit in Ninewells Hospital, no relationships were found between the expression of hyperactivated motility in a sample and the corresponding fertilisation rate at IVF. A hyperactivation assay based around the conditions used in this experiment would, therefore, be unable to predict either fertilisation success or failure. It would seem that there is no clinical advantage to using such a screen as a pre-IVF prognostic indicator in this setting.

Chapter 8: Effect of modulators of store-operated calcium entry on hyperactivation

8.1 Introduction

A wealth of evidence now points to the existence of a motility-controlling Ca^{2+} store located at the base of the sperm flagellum in mammals (discussed in Chapter 1). Sensitive to the pharmacological manipulation of ryanodine receptors, which have been localised to the midpiece region, it appears that in humans this store may therefore be opened by CICR (Harper *et al.,* 2004; Lefievre *et al.,* 2007). How the initial Ca^{2+} stimulus is initiated is unclear (Costello *et al.*, 2009). Mobilisation of the store can be noted upon application of progesterone and 4-AP to human sperm (Bedu-Addo *et al.*, 2008).

It is unlikely that the action of store mobilisation alone could induce any functionally significant rise in intracellular Ca^{2+}, nor the sustained elevation of Ca^{2+} which is particularly apparent upon application of 4-AP (Gu *et al.*, 2004). For example, in nominally Ca^{2+}-free medium , emptying Ca^{2+} storage organelles using bis-phenol or thapsigargin produces only a small, transient rise in $[Ca^{2+}]_i$ (Blackmore, 1993; Harper *et al.*, 2005; Espino *et al.*, 2009). With respect to motility, it has also been demonstrated that the Ca^{2+} directly mobilised from the store can only account for the initiation of hyperactivation, and that an external calcium source is required to maintain it (Bedu-Addo *et al.*, 2008).

The promotion of Ca^{2+} influx via CatSper has recently been identified as one the principal roles of progesterone in human sperm. However, blocking CatSper current entirely, using the channel blocker NNC55-0396, only partially inhibited the progesterone-induced initial Ca^{2+} transient (Strunker *et al.*, 2011). This indicates that a significant proportion of the Ca^{2+} which enters the cytosol following the application of progesterone does not come through CatSper.

In somatic cells, 4-AP causes mobilisation of $Ca^{2+}$from intracellular stores followed by Ca^{2+} influx through plasma membrane channels (Grimaldi *et al.,* 2001). This effect is termed store-operated calcium entry (SOCE), and a number of studies have indicated that it is active in human sperm (Williams and Ford, 2003; Espino *et al.*, 2009).

SOCE is a mechanism by which depletion of intracellular Ca^{2+} stores promotes the opening of 'store-operated' calcium channels (SOCC) in the plasma membrane. Whilst the primary evolutionary function of SOCE may have been the specific refilling of depleted storage organelles in order to maintain intracellular calcium homeostasis, it is a mechanism which can also play a role in the transduction of signals arising extracellularly (Putney, 1986; Putney, 2004). An agonist with the ability to mobilise intracellular stores can therefore elicit a prolonged Ca^{2+} signal in the cytoplasm.

The 'toolkit' for SOCE is made up of Ca^{2+} sensors and channels, with the key proteins identified in somatic cells being those belonging to the families STIM, Orai and TRPC (Parekh and Putney, 2005; Abramowitz and Birnbaumer, 2009; Costello *et al.*, 2009; Varnai *et al.*, 2009). STIM proteins serve as store-localised Ca^{2+} concentration sensors. They work in concert with Orais, and possibly also TRPCs, in the plasma membrane in order to promote the entry of extracellular calcium ions. The mechanism of action of SOCE is depicted in the diagram Figure 8.1.

Stimulus causes opening of Ca^{2+}-permeable channels on membrane of storage organelle

↓

Ca^{2+} effluxes from store

↓

STIM1, also located on membrane of store, senses store Ca^{2+} depletion

↓

STIM1 communicates with plasma membrane by redistributing into puncta

↓

Ca^{2+}-permeable plasma membrane channels formed from Orai (and possibly TRPC) proteins open

↓

Extracellular Ca^{2+} enters the cytosol

↓

Store is refilled by SERCA/SPCA and Ca^{2+} can act as a second messenger

↓

Process can be repeated

Figure 8.1: SOCE mode of operation. Adapted from information contained in (Parekh and Putney, 2005; Cahalan, 2009; Varnai *et al.*, 2009).

In humans, Western blotting and immunolocalisation have indicated that STIM and Orai proteins are expressed in human sperm. Orai 1 and 2 can be detected in the flagellum (Costello *et al.*, 2009), and STIM1 in the midpiece (Figure 8.2).

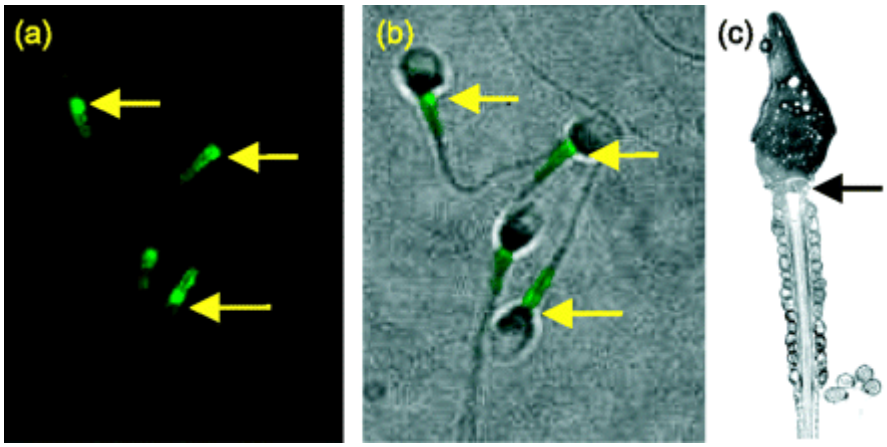

Figure 8.2: STIM1 expression in human sperm. From (Costello *et al.,* 2009). Images (a) and (b) show the immunolocalisation of STIM1 to the neck/midpiece of human sperm. The brightest spot is located in the region of the RNE (arrow in image (c)).

The movement of Ca^{2+} into the cell through plasma membrane channels involved in SOCE is termed 'I_{CRAC}' (CRAC = Ca^{2+}-release-activated calcium), and it can be manipulated using a number of pharmacological compounds in somatic cells. By modifying the interactions between the store-bound proteins and those at the plasma membrane, 2-aminoethoxydiphenyl borate (2-APB) potentiates STIM1-Orai1 mediated I_{CRAC} at low concentrations, that is, under 10µM, and inhibits it at higher concentrations, that is, up to 50µM (Wang *et al.*, 2009). At a concentration of 20-30µM, the imidazole SKF-96365 has also been shown to inhibit SOCE and I_{CRAC} in a number of different cell lines, by a mechanism yet to be fully elucidated (Prakriya and Lewis, 2002; Varnai *et al.*, 2009).

Both 2-APB and SKF-96365 have been shown to influence progesterone-induced Ca^{2+} signalling in human sperm. Recently, single-cell imaging experiments indicated that a 200s pre-incubation with 5µM 2-APB enhanced the Ca^{2+} transient induced by 3µM progesterone (Lefievre, 2011). This evidence suggests that a form of SOCE influenced by 5µM 2-APB is acting to promote the initial Ca^{2+} transient, in parallel with Ca^{2+} influx through CatSper (Strunker *et al.*, 2011). In contrast to these results, Park *et al.,* 2011, noted that inhibitory

concentrations of 2-APB and SKF-96365 (both 50µM) affected the smaller, sustained, midpiece Ca^{2+} signal (either a plateau or oscillations) in human sperm preparations.

Whilst Ca^{2+} signalling data such as that outlined above clearly implicates SOCE in the alteration of sperm motility patterns, there is little evidence on the influences of modulators of SOCE on hyperactivation directly. Therefore, the effects of 2-APB and SKF-96365 on hyperactivation, as measured by CASA, were investigated.

8.2 Experimental procedures

The sperm samples used in this chapter originated from research donors, all of whose ejaculate quality was within the normal range for concentration and motility according to WHO criteria as of 2009 (WHO, 1999). Sperm preparation methods and the assessment of hyperactivation using CASA are detailed in Chapter 4. Following preparation, sperm cells were resuspended in CM, the recipe for which is also presented Chapter 4. A number of experiments were carried out in order to monitor the effects of an agent known to potentiate SOCE, that is, 5µM 2-APB, and those known to have inhibitory effects on SOCE, namely, 50µM 2-APB and 25µM SKF-96365. Table 8.1 details the variables monitored in each.

Experiment	Aliquot 1	Aliquot 2	Aliquot 3	Aliquot 4	Aliquot 5	Aliquot 6
1	Control	3.6µM progesterone	2mM 4-AP	50µM 2-APB	3.6µM progesterone + 50µM 2-APB	2mM 4-AP + 50µM 2-APB
2	Control	3.6µM progesterone	2mM 4-AP	5µM 2-APB	3.6µM progesterone + 5µM 2-APB	2mM 4-AP + 5µM 2-APB
3	Control	3.6µM progesterone	2mM 4-AP	25µM SKF-96365	3.6µM progesterone + 25µM SKF-96365	2mM 4-AP + 25µM SKF-96365

Table 8.1: Pharmacological manipulations performed in order to test the role of putative modulators of SOCE on hyperactivation. For each experiment, sperm samples were divided into 6 aliquots. Drugs were only added immediately prior to CASA assessment, and aspirated four or five times before slide-loading to ensure homogenous distribution of both the drug and the cells. For aliquots 5 and 6 in each case, both drugs were added simultaneously, however, adding them sequentially (in either order), or pre-incubating (for two minutes) with one before adding the other, produced identical results to those depicted in section 8.4 below.

8.3 Statistics

No data set had a distribution which was significantly different to a normal distribution (determined using Kolmorogov-Smirnoff test), therefore paired sample *t*-tests were used to identify differences between variables in every case. Statistical tests were carried out using SPSS verison 17.

8.4 Results

Under these experimental conditions 5µM 2-APB, 50µM 2-APB and 25µM SKF-96365 all failed to induce any significant differences in hyperactivation expression (Figures 8.3, 8.4 and 8.5). This was the case both when each of the SOCE modulators was added to 'control' sperm, and when their effects were monitored in the presence of progesterone and 4-AP. The results displayed represent the mean values calculated across seven samples (from

seven donors) in each case, as this was the only way in which statistical significance in a population could be examined. With respect to each individual sample recorded in the presence or absence of 2-APB or SKF-96365, a comparison of the standard deviations between the four counts of 200 cells indicated that the majority of values here were not 'different' from one another either. In fact, differences of a similar size can be observed when analysing the same preparation twice in succession (results not shown). 2-APB or SKF-96365 therefore did not influence hyperactivation in either a stimulatory or inhibitory manner under these experimental conditions. This was the case not only for hyperactivation, but in fact for all motility parameters recorded.

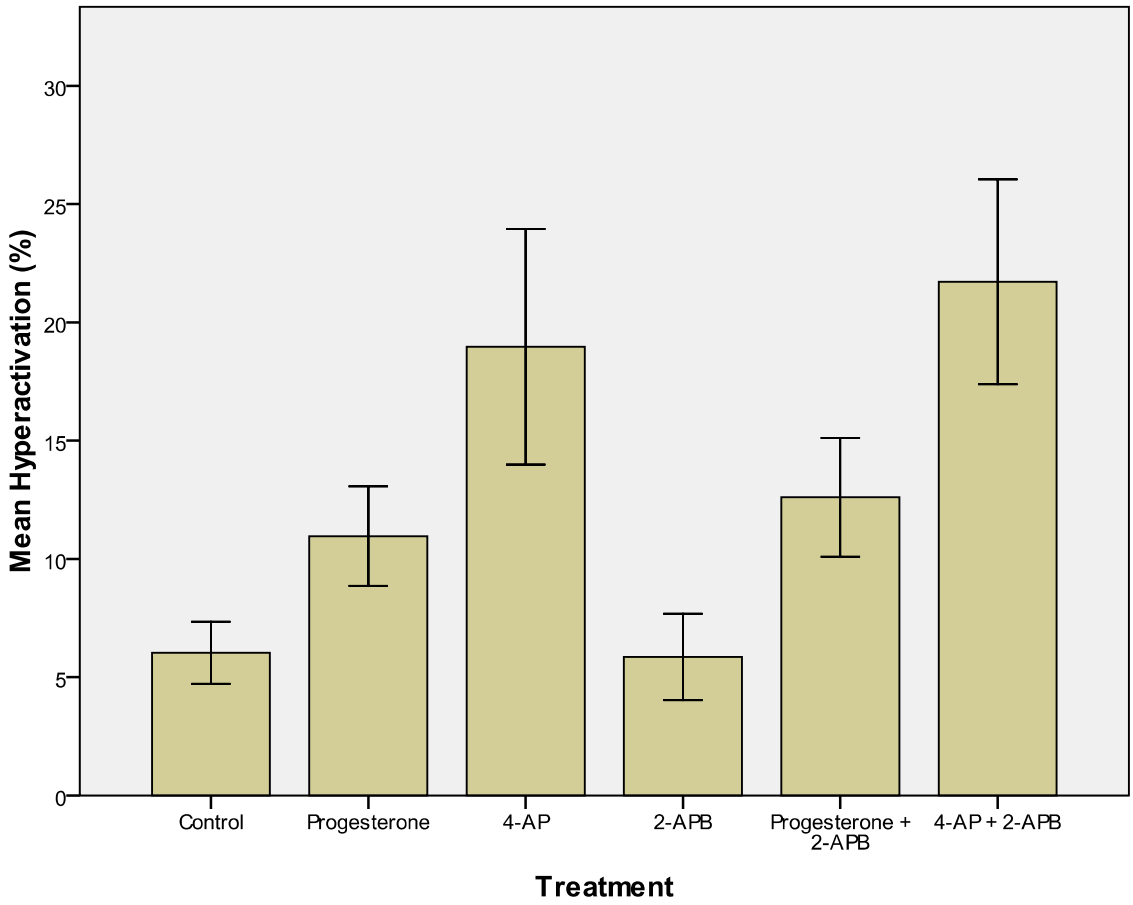

Figure 8.3: Effect of 5µM 2-APB on hyperactivation. Hyperactivation was first measured in the presence of progesterone and 4-AP. The effects on hyperactivation of 5µM 2-APB were then tested both in isolation and in conjunction with progesterone and 4-AP. Bars represent the average (plus or minus the standard error) of seven donor samples tested. The mean of the control group is significantly different to that of the progesterone group and the 4-AP treated group (*P*= 0.002 and *P*=0.018 respectively), as is the mean of the 2-APB treated group compared to that of the progesterone + 2-APB and 4-AP + 2-APB treated groups (*P*= 0.004 and *P*= 0.004 respectively). However, no significant differences arose following the addition of 2-APB to the control, progesterone or 4-AP treated groups.

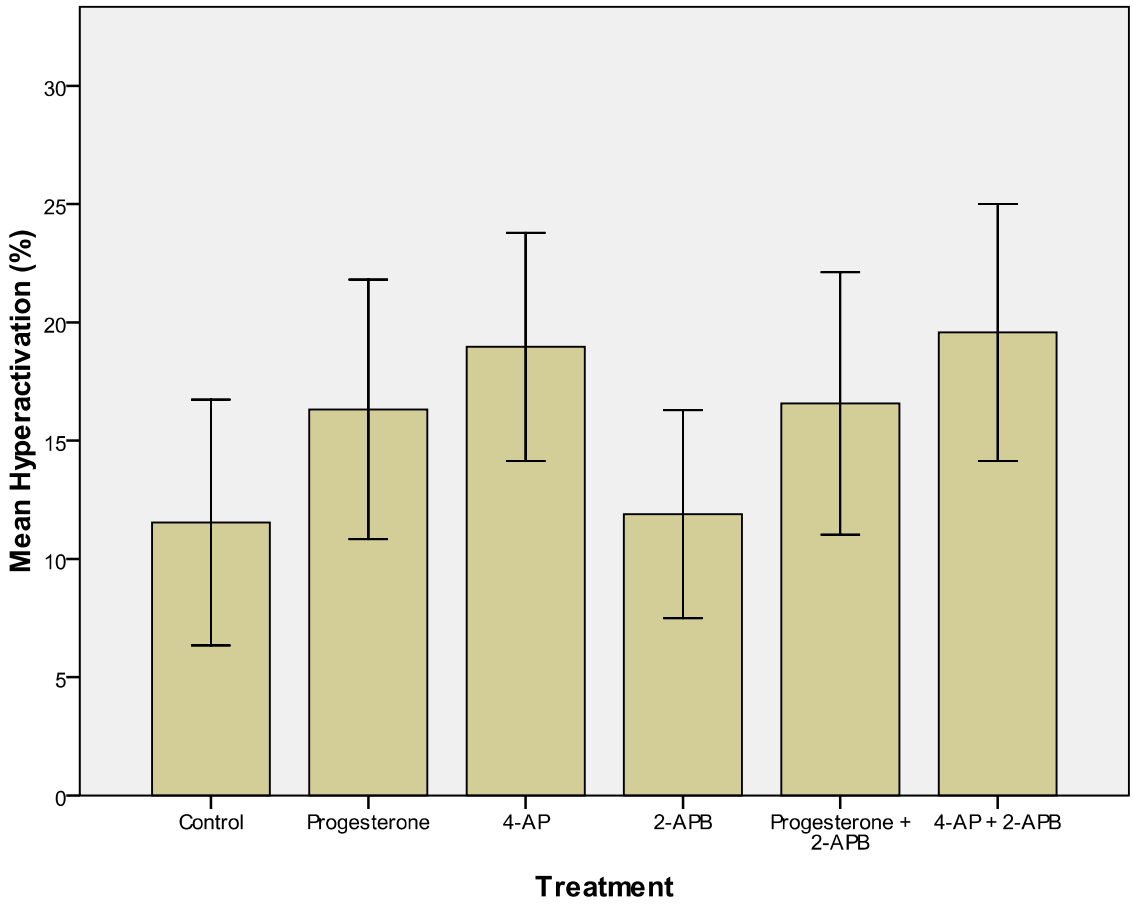

Figure 8.4: Effect of 50µM 2-APB on hyperactivation. Hyperactivation was first measured in the presence of progesterone and 4-AP. The effects on hyperactivation of 50µM 2-APB were then tested both in isolation and in conjunction with progesterone and 4-AP. Bars represent the average (plus or minus the standard error) of seven donor samples tested. The differences between the mean of the control group and the mean of the progesterone and 4-AP treated group are approaching levels of significance (*P*= 0.06 and *P*=0.07 respectively), and the mean of the 2-APB treated group is significantly different to that of the progesterone + 2-APB treated group and the 4-AP + 2-APB treated group (*P*= 0.041 and *P*= 0.014 respectively). No significant differences arose following the addition of 2-APB to the control, progesterone or 4-AP treated groups.

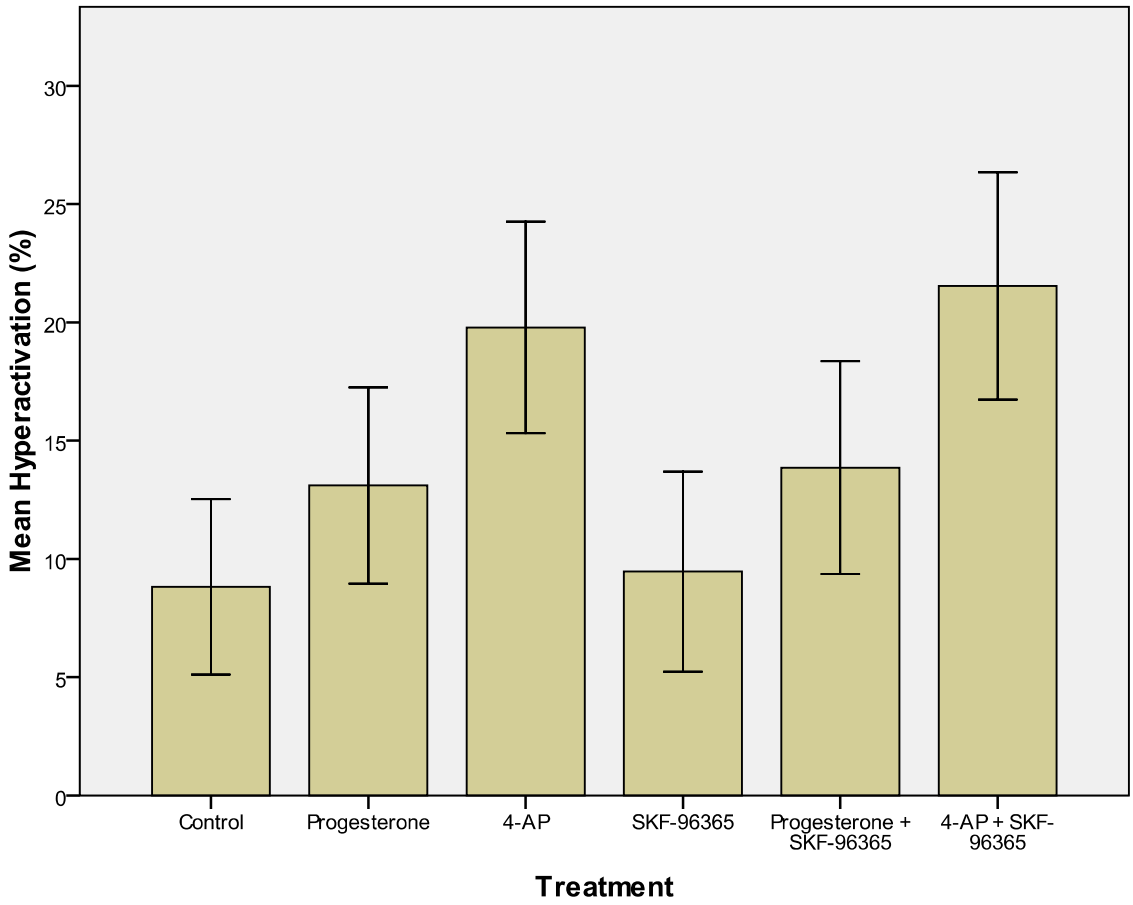

Figure 8.5: Effect of 25µM SKF-96365 on hyperactivation. Hyperactivation was first measured in the presence of progesterone and 4-AP. The effects on hyperactivation of 25µM SKF-96365 were then tested both in isolation and in conjunction with progesterone and 4-AP. Bars represent the average (plus or minus the standard error) of seven donor samples tested. The mean of the control group is significantly different to those of the progesterone group and the 4-AP treated group (*P*= 0.03 and *P*=0.001 respectively), as is the mean of the SKF-96365 treated group compared to those of the progesterone + SKF-96365 and 4-AP + SKF-96365 treated groups (*P*= 0.007 and *P*= 0.001 respectively). However, no significant differences arose following the addition of SKF-96365 to the control, progesterone or 4-AP treated groups.

8.5 Discussion

Given that previous evidence has indicated that SOCE is active in human sperm cells (Blackmore, 1993; Harper *et al.*, 2005; Espino *et al.*, 2009), and that both 2-APB and SKF-

96365 can influence Ca^{2+} signals (Lefievre, 2011; Park *et al.*, 2011), the findings here that SOCE was unaffected by such drugs could be considered surprising. With respect to SKF-96365, the results displayed here are contradictory to those of Castellano *et al.,* 2003, who found that the application of 20µM SKF-96365 to human sperm decreased the percentage overall motility by 30% following a one minute incubation. Interestingly, a similar level of inhibition was achieved by accident here, when a concentration ten times greater than this was applied (results not shown). The same authors' observation that 2-APB over a range of concentrations between 0.5 and 75µM was ineffective at altering motility was consistent with what was seen in this study.

It seems unlikely that flaws in any technical aspects of the experiments could be responsible for masking any effects of 2-APB and SKF-96365 on hyperactivation. For example, whilst the integrity of each drug preparation was not tested in other cells in which they have established activity, chemicals were purchased from a reliable source and stored according to manufacturer's instructions at all times. Moreover, once their inability to alter hyperactivation became apparent, fresh stock solutions were prepared in order to exclude the possibility of incorrect dilutions, etc. CASA analysis also seemed to be working correctly, as progesterone and 4-AP induced the same effects as they had done in the experiments carried out in Chapter 5.

There is a possibility that the agents used here did not alter the expression of hyperactivation because their effects on SOCE were confounded by the influences they can also exert on other pathways involving Ca^{2+} signalling. For example, whilst both 2-APB and SKF-96365 have been shown to influence STIM1-Orai1 mediated Ca^{2+} influx, neither acts specifically on this mechanism. For example, there is evidence that 2-APB can also act at

IP3 receptors, and inhibitory effects of SKF-96365 on TRPC's, VGCC's and potassium channels have been documented (Varnai *et al.*, 2009). However, the fact that previous investigators have observed very clear stimulatory and inhibitory effects on Ca^{2+} signals with these specific inhibitors at similar concentrations makes this less likely to be the reason that no effects on hyperactivation could be noted.

Therefore, assuming previous investigators are correct in their observations that SOCE is operational in human sperm, and that 2-APB and SKF-96365 do affect Ca^{2+} signals, what rationalisation could be offered as to why hyperactivation, or indeed any motion parameters, were unchanged by these modulators of SOCE under the experimental conditions applied here?

With respect to the comparison between the control (no drugs added) and the 2-APB treated groups in all three experiments, the lack of change in hyperactivation could possibility be explained by the fact that, in the absence of agents which specifically induce store mobilisation, the proportion of sperm that were undergoing store-mediated Ca^{2+} signalling in the first place was so small that modulation of SOCE by 2-APB and SKF-96365 was minimal. However, such a theory is put into question by the finding that 2-APB has the ability to elevate even resting $[Ca^{2+}]_i$ (Lefievre, 2011). In any case, what is certainly more unusual is that a similar lack of both stimulatory and inhibitory effects was also apparent even after stores were purposely mobilised using progesterone or 4-AP.

Considering progesterone treated samples, one research group have found that the amplitude of the immediate, transient rise in Ca^{2+} induced by 3µM progesterone is significantly increased in the presence of 2-APB (Lefievre, 2011). Moreover, this effect was

particularly pronounced in samples whose response to progesterone alone was relatively weak. These findings offer two potential explanations as to why 2-APB failed to influence hyperactivation in the presence of progesterone. Firstly, if the action of SOCE induced by progesterone is to modify the initial calcium transient, then it may be the case that any action was undetectable after the minute or so that it takes to obtain a reading following slide loading (discussed in Chapter 4). Secondly, the overall percentage increase in hyperactivation between the control and progesterone treated samples in these experiments was more than double what it was for the samples screened in Chapter 4: 53% (progesterone group significantly different to control) vs 22.7% (progesterone group not significantly different to control) respectively. This perhaps suggests that the sperm samples used in this chapter were particularly good progesterone responders in the first place, rendering any additional effects of 2-APB essentially negligible.

If SOCE acts to increase not the initial Ca^{2+} transient but the subsequent sustained phase of the progesterone-induced Ca^{2+} signal (Park *et al.*, 2011), then it is unusual that 2-APB and SKF-96365 did not modify hyperactivation, as, temporally, this phase should be able to be captured by CASA. The same can be said of the 4-AP treated samples. If the 4-AP-induced prolonged elevation of intracellular Ca^{2+} (which is accompanied by a sustained period of hyperactivation) is SOCE mediated, then why did drugs which influence SOCE not modify the hyperactivation response, perhaps in particular why were no inhibitory effects seen when levels were very high?

It is quite possible that, whilst 2-APB and SKF-96365 may be altering Ca^{2+} signalling patterns in human sperm, their effects are either not directly altering flagellar activity, or it is not possible to monitor these changes simply with CASA. Perhaps the observation of flagellar

movement in single cells at the exact point of application of the SOCE-influencing drug would help to establish whether or not SOCE truly was involved in promote motility changes (procedure for this described in Harper *et al.,* 2004). Further studies are needed, but if such work did indeed implicate SOCE in the generation/maintenance of hyperactivated motility, then specific dysfunction in store-mediated Ca^{2+} signalling may be identified as a novel cause of asthenozoospermia in a proportion of infertile men.

Chapter 9: General discussion and outlook

The primary goals of this project were: to find the most appropriate way of accurately recording hyperactivation, to explore the incidence of hyperactivation in men from different populations, to probe for direct links between hyperactivation and fertilising capacity, and to interrogate the mechanism of sperm motility signalling via stored Ca^{2+}.

A large amount of time was dedicated to understanding how CASA can be used to produce repeatable and reliable results with respect to the assessment of sperm motility parameters (detailed in Chapter 10). Therefore, when results emerged which supported the null hypothesis in Chapters 7 and 8 (i.e. that hyperactivation was not linked to fertilisation in vitro and that modulators of SOCE did not alter the expression of hyperactivation) it seems unlikely that inaccurate measurements were the cause of this.

With respect to Chapter 7, it was in fact recognised from the outset that the clinical potential of a pre-IVF hyperactivation screen would be limited. The field of andrology has changed a great deal since the original concept of sperm function testing emerged. Central to this change was, of course, the advent of ICSI (Palermo *et al.,* 1992). By circumventing the normal path to fertilisation entirely, ICSI essentially removed the need for sperm cells to 'function' at all; a nucleus containing genetic material was all that was required. Whilst in the 1980's and early 90's, the objective of assaying hyperactivation may have been primarily to explore ways in which to improve conventional IVF, for example, by using high insemination concentrations or by pre-treating sperm with heat or pharmacological agents, (Mortimer and Mortimer, 1992; Tournaye *et al.*, 1994; Fishel *et al.*, 1995; Chan *et al.*, 1998), as soon as ICSI became widespread, the need for such measures was negated. ICSI practice has, over the last fifteen years, 'exploded' in Europe, rising from 34.75% (of all treatment

cycles carried out) in 1997 to 63.3% in 2005 (Nyboe Andersen *et al.*, 2009); an increment which reflects not a rise in male infertility but a resistance amongst embryologists to 'risk' conventional IVF if they have any doubts regarding the quality of the sperm sample and its potential to generate a good fertilisation rate.

What this means in practice is that patient selection for IVF nowadays is very different to what it was during the time when the 'buzz' surrounding assays of sperm function led to the conduction of clinical hyperactivation studies such as those performed by Chan *et al.* in 1998, Guerin *et al,* in 1995 and Sukcharoen *et al.,* in 1995. For example, whereas in the 90's, men who presented with confounding factors such as poor/borderline progressive motility and/or morphology may have been included in such hyperactivation-IVF screens, these patients would now be directed straight to ICSI.

Arguably, sperm function tests could be considered *more* important in the current era of assisted reproductive technology, as embryologists now have to make a *choice* between IVF and ICSI, as opposed to only having the option of conventional insemination. However, these tests would really only be of use in the current era of assisted reproduction if they were able to pick out 'occult' hyperactivation pathologies, that is, those which existed against the background of an entirely normal semen analysis. Otherwise, assaying hyperactivation will be redundant, as the sample in question will have already been scheduled ICSI for another reason.

Therefore, whilst it was conceded that a hyperactivation screen may only have the potential to identify a small proportion of fertilisation failures, perhaps too few to be picked up in a sample size of less than 100, what was surprising was that even samples with

hyperactivation levels which measured close to zero under these conditions were able to fertilise eggs. The most likely explanation for this, from those offered in Section 7.5, is that the numbers of sperm used for insemination were too high for any deficiencies in hyperactivation to present a problem. However, it is certainly possible that samples displaying low levels of hyperactivation will face a genuine disadvantage under in vivo conditions, with fewer numbers of cells being able to reach even the surface of the oocyte. There may be scope, therefore, to examine the role of a hyperactivation screen in selecting, or more importantly, deselecting, couples for ovulation induction or IUI.

There is still some debate amongst the embryology community regarding the use/overuse of ICSI. However, unless any serious detrimental health aspects come to light in the future generations of ICSI babies, it will remain popular and effective tool in IVF. Embryologists, when faced with either carrying out an array of sperm function tests, or proceeding straight to ICSI, are likely to pick the latter.

Therefore, should the focus of clinical sperm research now be directed more towards improving the selection of sperm for ICSI? Whilst sperm functional capacity in ICSI is negligible, the integrity of the paternal genome has been shown to be contributory to not only fertilisation, but crucially, the developmental fate of the embryo (Avendano *et al.*, 2010; Lewis and Simon, 2010; Simon *et al.*, 2010). Abnormal sperm DNA can be take the form of chromosomal alterations such as aneuploidies, incomplete remodelling of chromatin during packaging, or strand breaks caused by oxidative damage. As men whose semen parameters are poor are more likely to produce sperm cells which have damaged DNA, in an ICSI cycle there is a greater chance of introducing a genetically flawed paternal genome into the oocyte (Oehninger and Kruger, 2007). Whilst assays which detect DNA

fragmentation, such as TUNEL, Comet and SCSA, or those which detect aneuploidy such as FISH, are useful for diagnosing male infertility and predicting IVF outcome (Simon *et al.*, 2011), they cannot help with the real time selection of genetically normal individual cells. Current practice of ICSI typically involves the selection of sperm at 400x magnification. However, with the use of specialised contrast optics and a magnification of over 6000x, it is now possible to examine live, motile sperm at the organelle level. Sperm selected using this method prior to their microinjection ('IMSI') have been shown to have less DNA fragmentation, and to produce fewer aneuploid embryos, than those which would otherwise have been selected for standard ICSI (Figueira Rde *et al.*, 2011; Wilding *et al.*, 2011). Correspondingly, results suggest that this results in significant increases in implantation and pregnancy rate (Berkovitz *et al.*, 2005; Setti *et al.*, 2010), perhaps particularly in men with poorest quality semen (Balaban *et al.*, 2011). There is another way in which sperm with fewer genetic aberrations may be selected for ICSI; a technique referred to as PICSI™. This method works on the principal that mature sperm are less likely to present with DNA fragmentation, incomplete chromatin remodelling or chromosomal abnormalities. As only mature sperm can effectively to bind to solid-state hyaluronic acid, the substance is used as an indicator for high intergrity sperm (Oehninger and Kruger, 2007). Such advances may therefore optimise treatment outcomes for both moderate and severe male factor patients, and even patients whose semen analysis is normal yet whose DNA status is compromised.

The presence of sperm DNA damage may actually have a specific connection to hyperactivation also. Sperm cells produce reactive oxygen species which are known to play a role in the induction of both hyperactivation and capacitation. However, this involves a critical balancing act between ROS generators and ROS scavengers, as the production of

too much ROS at an inappropriate time can have a detrimental effect on sperm function (De Jonge and Barratt, 2006). When hyperactivation occurs spontaneously, that is, in semen as opposed to in the female reproductive tract or a medium of similar chemical constituents, it may reflect the fact that the sample has a low ROS scavenging capacity (de Lamirande *et al.*, 1997). Cells in such an environment will clearly be under increased oxidative stress. This may not only affect their functional capacity, but also directly damage their DNA. In this respect, hyperactivation may serve as a marker with which to identify a sample at risk of having a higher proportion of damaged DNA. Whilst this would only be useful for diagnostic purposes at present, there may, in the future, be a way to treat and/or prevent the effects of free radical attack.

With respect to the elucidation of the signalling pathways responsible for the control of hyperactivation, such as the putative role of SOCE, more work is required. Understanding the nature of all aspects of sperm function has important implications for male fertility. For example, it may be possible to treat a proportion of sperm disorders therapeutically. If such breakthroughs could be made, it may diminish the need to consistently resort to ART, which is expensive, invasive, and whose long term effects on the health of future generations are unknown. Equally importantly, research into the workings the sperm cell will be crucial in the identification of drug targets which could be exploited by non-hormonal male contraceptives. Indeed, CatSper is being hailed as a candidate for such a role at present (Hildebrand *et al.*, 2010).

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Chapter 10: Appendix

10.1 Evolution of methods

From initial observations and the study of previous published work it was recognised that a number of variables may affect both the expression of hyperactivation in a sample and its measurement using CASA. Without careful control of these variables, making a reliable comparison of hyperactivation levels between samples, or before and after pharmacological manipulation of a sample, would be very difficult. Moreover, if, even after controlling all variables, repeatable and reliable results could not be attained, then it was acknowledged that the assessment of hyperactivation may be inherently too inaccurate to be used as a transferrable assay.

In order to adequately standardise the testing methods, and determine the optimal conditions under which hyperactivation should be assessed, the method outlined in the methods section was developed and optimised in the weeks prior to gathering any data from clinical samples.

The factors that could influence the expression and measurement of hyperactivation were proposed to be those outlined in Table 10.1.

Definite variables	Probable variables	Possible variables
Medium	Temperature	Concentration of sperm cells
CASA settings	Incubation period	Sperm preparation method
	Number of cells counted	

Table 10.1: Putative variables affecting the expression and measurement of hyperactivation.

Sperm samples produced by research donors were used to test the effects of each parameter in the table above, and these were varied independently.

10.1.1 Medium

As the expression of hyperactivation requires $H^+CO_3^-$, Ca^{2+} and glucose, the concentration at which these components are present in the extracellular medium is likely to have a crucial influence on its expression. Media designed to support capacitation may also be richer in other components which play a role in the initiation and maintenance of hyperactivation. They may also be more alkaline in pH, thereby potentiating CatSpers.

Clinical samples received from the embryology laboratory arrived in either Cook Sperm Medium™ (if the sample was used in conventional IVF) or Cook Gamete Buffer™ (if the sample was used in ICSI). Sperm Medium is bicarbonate-buffered, pH 7.5-7.8 when equilibrated with sufficient CO_2, and designed to support capacitative events. Gamete Buffer is HEPES based, held at pH 7.3-7.5, and is for use in a situation where capacitation of sperm is not required (e.g. in ICSI).

Hyperactivation levels in the two media were compared, whilst all other variables in Table 10.1 were kept constants. Results are shown in Figure 10.1.

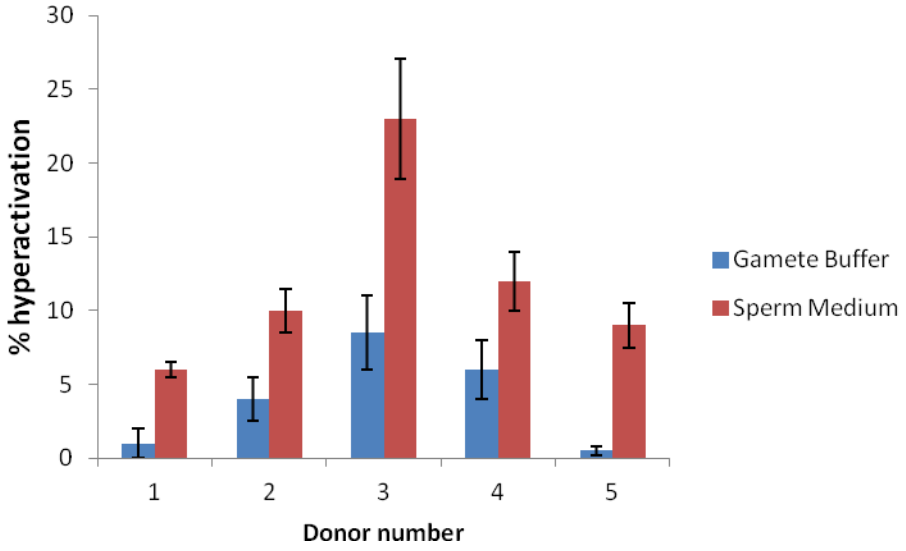

Figure 10.1: Hyperactivation in capacitating and non-capacitating clinical media. Error bars represent the standard deviation between four counts of 800 cells.

The results show unsurprisingly that the levels of hyperactivation were significantly greater (paired samples *t*-test, *P*= 0.01) in samples suspended in Sperm Medium compared to Gamete Buffer. Mirroring these results were those obtained for hSTF (capacitating) and NCB (non-capacitating), with the commercial media inducing only slightly elevated rates of hyperactivation (not significantly different) compared to their laboratory-synthesised counterparts (results not shown). Only like for like medium was used to monitor the effects of other variables on hyperactivation to exclude the possibility of extra variables being introduced in this way.

10.1.2 CASA settings

Image frame rate and number of frames recorded were standard for the CEROS machine. All other aspects of setup were checked every day prior to using the CASA, to ensure that they were kept constant throughout the data recording.

10.1.3 Temperature

Early studies of hyperactivation indicated that hamster sperm hyperactivated more readily at 37^{o}C than at room temperature (approx 24^{o}C) (Mahi and Yanagimachi, 1973). Conversely, another study carried out in 1989 suggested the opposite; that hyperactivation *decreased* when moving from 24^{o}C to 37^{o}C (Mack *et al.*, 1989). The authors proposed that it might due to an effect of temperature on calcium flux; however, this effect was also noted in this study when the hyperactivation algorithm was varied. Interestingly, if the VCL component of the hyperactivation algorithm is set too low, then cells that are simply struggling to swim forward, which results in the ALH value increasing, are incorrectly identified as hyperactivated (own observations).

When a microscope slide at room temperature is placed on the CASA heated stage there is a lag period before it equilibrates to 37^{o}C. Figure 10.2 depicts how long it takes for sperm on a 'cold' slide to express their maximal levels of hyperactivation. This time almost corresponds exactly to the time it takes for the stage to warm a slide to 37^{o}C. Pre-warming of the slide and cover slip negates the need for any lag period, and assessment can begin within 30s (results not shown). Therefore all microscope slides used in this experiment were pre-warmed, to ensure cells were only recorded at 37^{o}C.

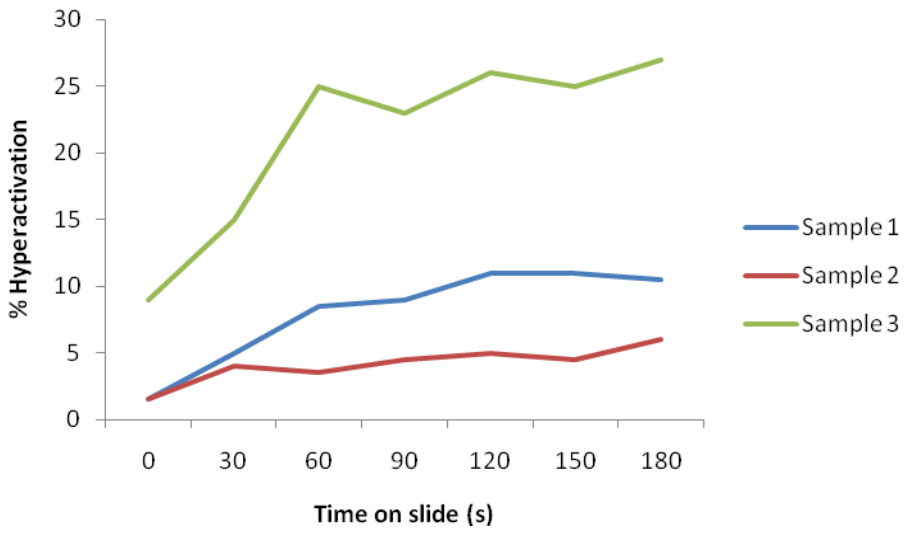

Figure 10.2: Effect of temperature on hyperactivation. The point at which hyperactivation levels start to plateau (around 120 seconds) corresponds with the time it takes for a cold slide to heat to 37^{o}C following its placement on a heated stage. All other variables in Table 10.1 were kept constant.

10.1.4 Incubation period

To become capacitated sperm must spend a period of time (thought to be anywhere between one and six hours) either in the female reproductive tract or in vitro in a capacitating medium. Whilst capacitation and hyperactivation may not share the same underlying signalling pathway, a prerequisite for observing the maximal levels of hyperactivation in a sample may also involve an incubation/priming period in medium. This was investigated by monitoring samples for hyperactivation levels at t=0, 2, 4 and 6 hours (Figure 10.3).

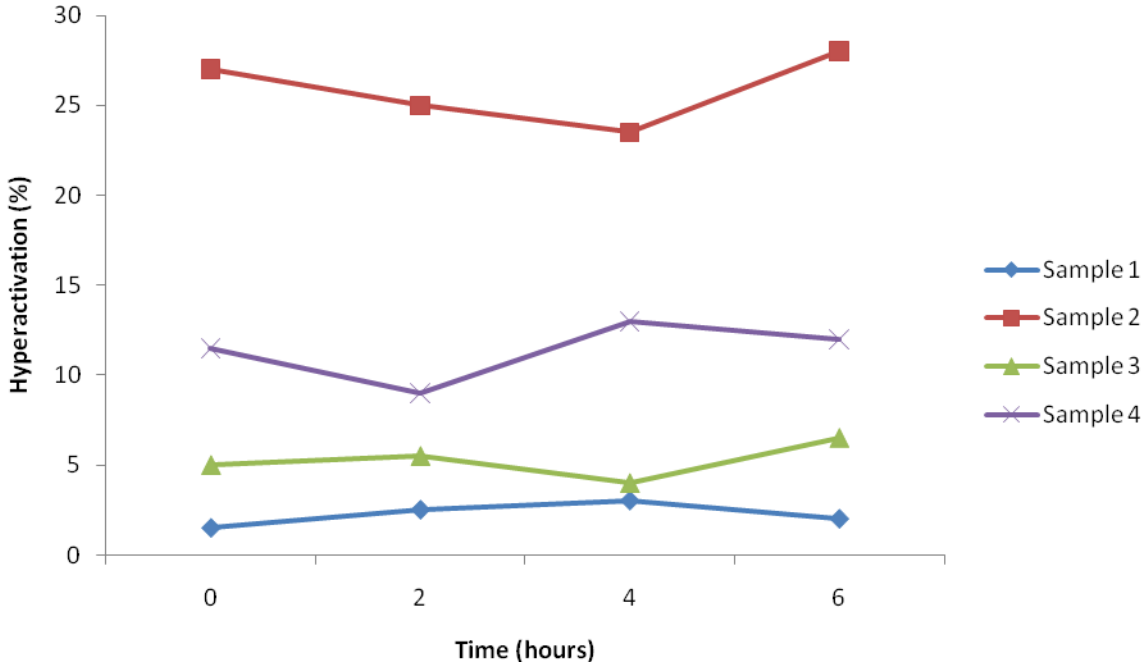

Figure 10.3: Variation of hyperactivation with time spent in capacitating medium. Results shown represent time incubated in commercial medium, that is Cook Sperm Medium™, however the results obtained for the home made medium (CM) mirrored these.

No statistical differences could be found between hyperactivation levels recorded at different time points (one-way ANOVA, *P*= 0.997). Speculatively, this suggests either that capacitation occurs very rapidly in vitro, or that capacitation is not an essential prerequisite for hyperactivation. In practice, this means that hyperactivation in samples can be compared as like for like regardless of their incubation time period. There may be a danger that sperm left for too long will start to either run out of energy or 'burn out' and for this reason measurement time was restricted to within eight hours of preparation, as no deterioration was noted by this time point (results not shown).

10.1.5 Number of cells counted

In counting cells to assess what proportion of a sample exhibits a specific feature (e.g. in terms of motility, morphology or vitality) it has been highlighted that duplicate counts of only 100 cells result in large confidence intervals between values (WHO, 1999). The significance of this may even be enhanced when dealing with the measurement of hyperactivation because it is a parameter which, unlike e.g. morphology, is not fixed, but biphasic. Figure 10.4 highlights that counting only 100 cells, as previous investigators have done, may not generate an accurate value for the true percentage of hyperactivation in a sample. Since CASA assessment is so quick, there seems no reason not to count 800 cells.

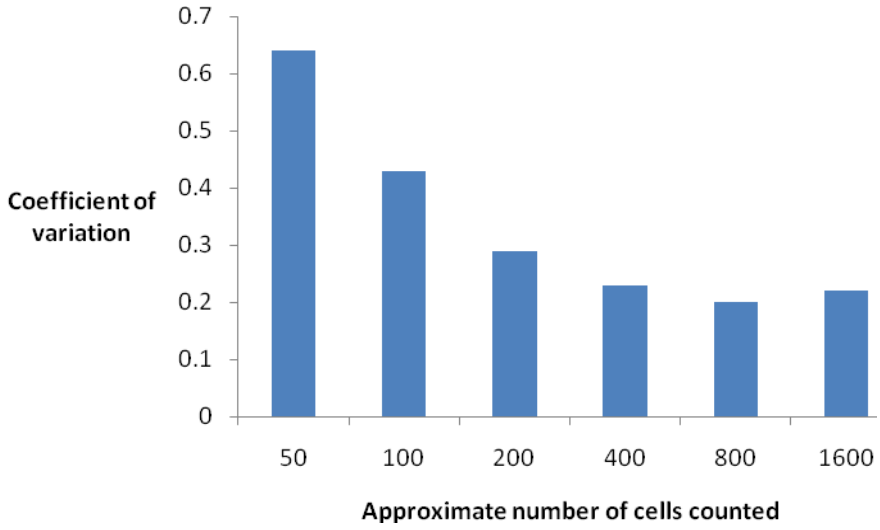

Figure 10.4: Variation in hyperactivation based on number of cells counted. The bars represent the average co-efficient of variation obtained from when CASA was used to make duplicate assessments of hyperactivation using different cell counts of one sperm sample.

10.1.6 Concentration

The tracking of sperm heads using CASA could be complicated by having too many cells present in each frame, for example if separate cell trajectories became so close together

that the system became unable to identify an individual sperm's trajectory because there were other cells in its 'search radius' (Mortimer, 1997). This may lead to track points being lost and cells omitted from the analysis. In addition, it seems possible that in densely populated samples may behave differently to sperm which are more spaced out, for example as a result of greater net numbers of reactive oxygen species that may be generated (de Lamirande *et al.*, 1997).

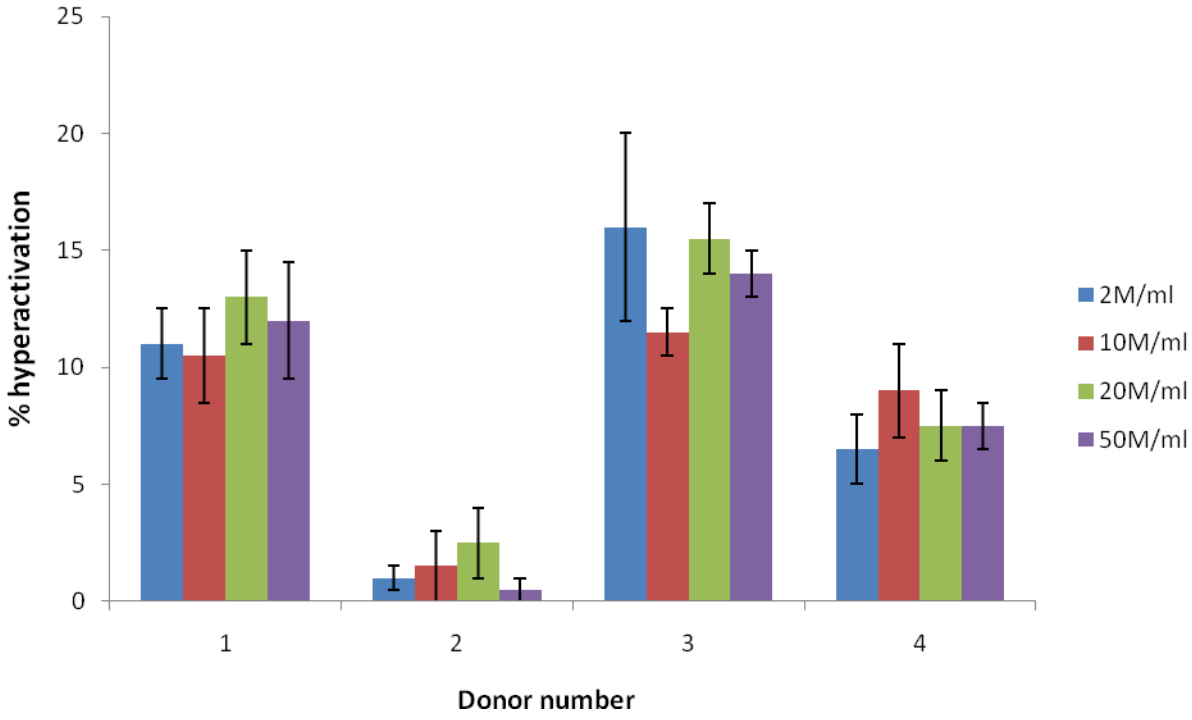

Figure 10.5: Vatiation in hyperactivation with sperm cell concentration. Results shown are the levels of hyperactivation in four donor samples tested at different sperm cell concentrations. Error bars represent standard deviation between four counts of 800 cells.

Figure 10.5 demonstrates that no significant differences (one-way ANOVA, P=0.984) were found when assaying hyperactivation at different concentrations. 10-20M/ml was therefore chosen as the optimal concentration at which to measure hyperactivation as it will permit the rapid assesment of 800 cells, whilst still maxisiming the use of each sperm sample.

10.1.7 Sperm preparation method

As density gradient centrifugation is known to produce a higher yield of motile cells than the swim up technique (Bjorndahl *et al.*, 2010) (and does not cause any damage to sperm cells) this method of preparation was kept constant throughout the data gathering.

10.2 Consent form for patient/donor participation in research

ASSISTED CONCEPTION UNIT

NHS TAYSIDE

WARD 35

NINEWELLS HOSPITAL

DUNDEE DD1 9SY

Direct line (01382) 632111

Fax (01382) 633853

CONSENT FORM FOR PATIENTS/DONORS

[producing extra semen samples]

Title of research: Understanding the regulation of human sperm function and the development of novel treatments for male infertility.

First of all we would like to thank you very much for taking part in our research project.

The aim of this study is to understand how a sperm cell is activated in response to secretions from the female tract - progesterone and nitric oxide and to understand if this activation is abnormal in some men. In addition we would like to test enzyme inhibitors to see if we can enhance sperm motility and hope that in the future we may be able to develop drugs which may be able to improve IVF success.

You may decline to take part, or withdraw at any time without this affecting, in any way, your treatment and care now or in the future.

I have fully understood what will be involved in the project. This study involves me producing a semen (sperm) sample by masturbation in the Assisted Conception Unit or by arrangement at home, for the research purposes of the project. In the future there may be requests for further semen samples.

Signed…….

Name (block capitals)……..

Date……

Witnessed……(name)……Signature

If you have any further queries or questions you can contact either: Mr Steven Mansell (01382 660111 ext. 33605) or Nurse Evelyn Barratt, e.barratt@dundee.ac.uk